Host plant toxicity, stenophagy and evolutionary radiation in phytophagous insects: genus *Ceratitis* (Diptera: Tephritidae) as ecological model

Gastplant-toxiciteit, stenofagie en evolutionaire radiatie in fytofage insecten: het genus *Ceratitis* (Diptera: Tephritidae) als ecologisch model

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Section I: Introduction

Collection site, Naivasha, Kenya © Nathalie Erbout
A journey of a 1000 miles, begins with taking the first step (Lao Tse)

Chapter 1.1

General Introduction

Today's biodiversity is the manifestation of a long evolutionary process whereby many species became extinct and new ones arose (Wilson, 1992). Insects comprise a significant portion of the earth's biodiversity with an estimated three to ten million species. They occur in many biogeographical regions and ecological zones, from the Arctic Circle (Danks, 1981) to Antarctica (Block, 1992) to the tropics (Godfray et al., 1999), and their (relatively) high level of mobility enables them to disperse long distances, invade previously unoccupied regions, and occupy all conceivable (micro)habitats where they often play key functional roles (Kenis et al., 2009). Whereas some species are major agricultural pests and disease vectors, many others contribute positively to the world's economy through the provision of ecosystem services and goods (Weisser and Siemann, 2004), for instance as decomposers, consumers, pollinators, predators and parasites (Diaz et al., 2006).

Nearly half of the current-day insect species display a phytophagous lifestyle (Novotny et al., 2002; Ødegaard, 2006). Phytophagy, however, does not occur to the same extent in all insect groups. Whereas some of the 32 insect orders are almost exclusively phytophagous (e.g. Lepidoptera, Hemiptera, Coleoptera and Diptera), herbivory occurs less frequently or is even absent from others (e.g. Siphonaptera, Odonata) (Schoonhoven et al., 2006). Recent studies identified several complex relationships between phytophagous insects and plants, including adaptations of life-cycle strategies (Lewinsohn et al., 2005). As insect herbivores also play a key role in the course and rate of host plant succession, studies of insect-plant associations significantly advanced our understanding of the ecological and evolutionary processes that underlie biodiversity (Whitham et al., 2006). Despite the fact that phytophagous insects have potentially access to a large supply of resources, most species are highly host specific (Jaenike, 1990), i.e. less than 10% of all phytophagous insects feed on

plants from more than one family (Bernays, 1991). Studies on host-use patterns have shown that several factors, including plant chemistry, physiological efficiency, performance trade-offs on different hosts, host availability and oviposition choice (Singer, 1983) may be important in determining host range. To obtain a better understanding of (the evolutionary dynamics of) insect-host plant relationships, multiple ecological factors and trade-offs should be studied, preferably through a multidisciplinary approach (Bernays, 1991).

It is generally assumed that the evolution of insect-host relationships has been largely triggered by the chemistry of insect host plants (Ehrlich and Raven, 1964), given the large and taxon-specific diversity of so-called secondary compounds or secondary metabolites, the relatively narrow host range of many phytophagous insects, and the fact that plant secondary metabolites, which play an important role in restricting phytophagous insect diets, are toxic to some species but appear harmless to others (Fraenkel, 1959). Some plant groups pose apparent chemical barriers to potential herbivore insects, and restrict accessibility to relatively few insect lineages that are possibly preadapted by their initial use of chemically similar or related host plants (Becerra, 1997). To cope with the different classes of secondary plant compounds, phytophagous insects must possess physiological response mechanisms that enable them to detect and reject potentially toxic compounds in plants and to override their aversive response selectively to harmless compounds (Glendinning, 2002). At first instance, the aversive response causes insects to limit the intake of a particular food until they can assess its potential toxicity. The second phase of the coping process involves selectively overriding the adverse response to relatively harmless compounds through the use of detoxification mechanisms. Phytophagous insects that fail to execute such mechanisms will be exposed to stress when encountering such toxic environments. In a pre-adult stage, environmental stress is believed to impose energetic costs to developing organisms (Sibly and Calow, 1989; Baillieul et al., 1996), thereby reducing their level of developmental homeostasis and increasing their level of developmental instability, i.e. ability to buffer developmental pathways against random perturbations of genetic or environmental origin (Clarke, 1993). The

degree of developmental stability of an organism (or population) is most commonly estimated by its level of fluctuating asymmetry (hereafter FA) (Van Valen, 1962), defined as the magnitude of small random deviations from perfect symmetry in one or more bilateral traits (Palmer, 1994). FA is believed to relate positively to developmental stress of environmental or genetic origin, and negatively to quality or fitness (Leung and Forbes, 1997).

Whether, and to what extent, secondary plant metabolites triggered the evolution of host plant specialization in phytophagous insects is still much debated (Jaenike, 1990). Species may either evolve into host plant specialists by physiologically adapting to toxic host plants that were previously unexploited, or into host plant generalists at the cost of lower feeding success on any particular hosts, but with a wider range of available hosts (Futuyma and Moreno, 1988). Yet, no single variable (including plant chemistry) has been identified that significantly explains the observed variation in host plant use (Futuyma and Moreno, 1988), and there is no consistency in physiological benefits of one strategy over another. Hence, it has become evident that host plant use is influenced by both ecological factors, such as host plant availability and selection, and physiological constraints (Singer, 1983), emphasizing the need for a multidisciplinary approach when studying host plant specialization and the variety of factors that may influence its evolution.

Study objectives

Using a multidisciplinary approach, my doctoral study aims to describe the biogeography and phylogeny of host plant specialisation in phytophagous fruit flies of the genus *Ceratitis* (Diptera, Tephritidae) and to unravel mechanisms that underlie the evolutionary radiation of stenophagous (specialist) clades within this genus (see De Meyer, 2000, 2001). Representatives of the genus *Ceratitis* demonstrate both specialist and generalist strategies. Based on the observation that host plants of stenophagous species often contain high levels of toxic secondary metabolites (Coates and Palgrave,

1983; Mabberley, 1997) that may curb or prevent larval development in absence of tolerance mechanisms, results of this doctoral study are discussed against the hypothesis that ancestral species of present day stenophagous *Ceratitis* clades developed a mechanism to cope with toxic secondary metabolites, thereby creating opportunities for infestation of previously (by them) unoccupied hosts, for host plant shifts, and for cladogenesis.

Tephritidae (Diptera)

The Tephritidae is one of the larger globally distributed families of Diptera, with more than 4500 described species in more than 500 genera (Norrbom et al., 1999). Members of the fly family Tephritidae have been the subject of extensive biological investigation, representing a significant evolutionary and economic fauna. Tephritid research has contributed to our general understanding of basic biological problems as well as pest control. Fossil evidence of tephritids is rare (Norrbom, 1994). Three species are known from compression fossils and two species from amber (Norrbom, 1994). The oldest specimen, *Protortalotrypeta grimaldii*, is from Dominican amber and is estimated to be 25 million years old (Norrbom, 1994). Freidberg (1991) described a *Ceratitis* sp., possibly *C. rosa*, from amber from Tanzania that is about 3 million years old. The Tephritidae are considered to have originated in the paleotropics, presumably post-Gondwanan, because of the high species diversity encountered there (Norrbom, 1994). The zoogeography of extant species and fossil evidence suggest that the genera of the subfamilies Trypetinae and Dacinae represent the ancestral lineages of the family, whereas the Tephritinae appeared later with species diversity concentrated in the subtropical and temperate regions of the world (Norrbom, 1994).

The Tephritidae are not uniformly phytophagous, it also includes many saprophagous species and gall formers (Korneyev, 1999). Among the phytophagous species are frugivorous and nonfrugivorous species. The nonfrugivorous appellation refers to species of tephritids whose larval foods are plant parts other than fleshy fruits

(Zwölfer, 1983). Most nonfrugivorous tephritids are not economically important, but few species attack cultivated plant species (see White and Elson-Harris, 1992). For frugivorous fruit flies on the other hand, larval foods consist of fruits. The lifecycle of frugivorous tephritids comprises six stages: egg, three instar stages, pupa and adult. After developing into the third instar stage, larvae leave the host fruits and jump to the soil where pupation and subsequent emergence take place (Fletcher, 1989).

Genus Ceratitis (Diptera, Tephritidae)

The frugivorous fruit fly genus *Ceratitis* MacLeay belongs to the tribe Ceratidini, subfamily Dacinae (Smith et al., 2002) and is a composite of six subgenera (*Ceratitis sensu stricto, Ceratalaspis* Hancock, *Pardalaspis* Bezzi, *Pterandrus* Bezzi, *Hoplolophomyia* Bezzi, and *Acropteromma* Bezzi), comprising 95 described species (De Meyer, 2005). Its taxonomic position and relationship to other Tephritidae was outlined by De Meyer (1996, 1998). Although native to the Afrotropical mainland and islands of the western Indian Ocean, a number of *Ceratitis* species are adventive elsewhere, and the genus includes economically significant agricultural pests that are considered potential invasive species (White & Elson-Harris, 1992). Like in other tephritids, *Ceratitis* biology is associated with host plants that provide food for developing larvae and putative mating sites for adults. Representatives of this genus are true frugivorous in that eggs are deposited in - and larvae solely feed on- fruits. The host range of *Ceratitis* flies ranges from extreme generalist (polyphagous), infesting plant species from more than 20 host-pant families, to very specialized, infesting solely few particular host-plant families (oligophagous), one host-plant genus (stenophagous) or one host-plant species (monophagous). A total of 48 host families are recorded for this genus in Africa, comprising more than 60 genera (http://projects.bebif.be/enbi/fruitfly). Among these genera, a number are known to comprise insecticidal secondary metabolites, some of which are plant species restricted (Coates and Palgrave, 1983; Mabberley, 1997). These secondary plant metabolites are non-nutritional chemicals that affect the growth, health or behaviour of other species

(Whittaker, 1970), in this case the fruit fly larvae. As secondary metabolites are produced from universally present precursors, a classification derived from their biosynthetic pathway appears to be suitable for most cases (Buchanan *et al.*, 2000). A simplified classification distinguishes nitrogen-containing compounds, terpenoids, phenolic compounds and acetylenic compounds. An overview of some main secondary plant metabolites of host plant families and genera of *Ceratitis* fruit flies is shown in table 1.

Figure 1: Picture of *Ceratitis anonae*

Table 1. Main secondary metabolites (MSM) from hosts from stenophagous *Ceratitis* flies (Trease & Evans, 2002).

Host family	***Family MSM***	***Host genus***	***Genus MSM***	***Infesting flies***	***References toxicity***
Solanaceae	glycoalkaloids	*Solanum*	solanine	*C. aliena* *C. turneri* *C. venusta* *C. marriotti*	Nema et al, 2008 insecticidal
Loganiaceae	indole alkaloids	*Stychnos*	strychnine	*C. lobata* *C. curvata* *C. pedestris*	Yin et al, 2007 Cytotoxicity
Sterculiaceae	triterpenoids	*Cola*	caffein	*C. acicularis* *C. penicillata* *C. colae*	trease & evans p388 neurotoxin
Rubiaceae	Indole alkaloids	*Coffea*	caffein	*C. colae*	trease & evans p388 neurotoxin
		Oxyanthus	cyanogenic glycosides	*C. cornuta*	Rockenbach et al, 1992 poisonous HCN
Podocarpaceae	norditerpene & bisnorditerpene dilactones	*Podocarpaceae*	norditerpene & bisnorditerpene dilactones	*C. gravinotata* *C. podocarpi*	kubo et al 1985; Singh et al, 1979 insect growth inhibitor
Apocynaceae	tryptophane-derived alkaloids	*Tabernaemontana*	Strictosidine glucosidase	*C. edwardsi* *C. cuthbertsoni* *C. millicentae* *C. lepida*	Luijendijk et al, 1996 Antifeedant activity
	triterpenoids	*Acokanthera*	ouabain	*C. simi*	Torrie et al, 2004; Abassy et al, 1977 antifeedant
Rutaceae	indole alkaloids	*Vepris*	Furoquinoline alkaloids	*C. cristata* *C. argenteobrunea*	Trease & Evans, 2002; Brader et al, 1996 Insecticidal effect
Ebenaceae	naphtoquinones	*Diospyros*	ursolic & oleanolic acid	*C. pennitibialis*	Mallavadhani et al, 2003 Antifeedant; insect growth inhibitor
Moraceae	cardenolides	*Antiaris*	ouabain	*C. flexuosa*	Carter et al, 1997a, 1997b; Gan et al 2009 cytotoxicity

Thesis Outline

Overall structure

The doctoral thesis consists of four consecutive sections. **A first section** introduces and reviews the general ecological and evolutionary processes underlying insect-host relationships in phytophagous insects. **A second section** describes biogeographic and phylogenetic patterns of host plant use by *Ceratitis* fruit flies. **A third section** introduces the use of an individual-based estimator of developmental stress (fluctuating asymmetry), validates the assumption that stenophagous and polyphagous host plants differ in concentration of toxic secondary metabolites, and experimentally tests the hypothesis that host plant toxicity imposes developmental stress in polyphagous *Ceratitis* species. **A final, fourth section** integrates the most important research findings of the different case studies and discusses perspectives for further research.

Individual manuscripts

In **manuscript 1.2**, we review existing literature to confer mechanisms that determine host plant choice and selection in phytophagous insects, to discuss which processes are known to enhance specialization, and to evaluate the role of chemical mediation in insect-host relationships.

In **manuscript 2.1**, we study the biogeographical distribution of African fruit infesting fruit flies and focus on variation in fruit fly specialization at the subbiome level.

In **manuscript 2.2**, we reconstruct the phylogeny of 49 species of *Ceratitis* fruit flies, representing a large part of the known stenophagous species of the major species groups within the genus, using molecular data from one nuclear and two mitochondrial encoding genes. We investigate the evolution of host-plant specialization along different recognized monophyletic clades and reconstruct the ancestral character states for host plant relationships.

In **manuscript 3.1**, which is a methodological chapter, we test the use of fluctuating asymmetry as an individual-based marker of developmental stress by comparing asymmetry levels in two parental *Ceratitis* species, *Ceratitis fasciventris* and *Ceratitis rosa*, and their hybrid offspring.

In **manuscript 3.2**, we use HPLC analysis to compare the concentration of two toxic secondary metabolites, α-chaconine and α-solanine, in a host plant of stenophagous (*Solanum anguivi*) and polyphagous (*Solanum mauritianum*) fruit flies.

In **manuscript 3.3**, we test if, and to what extent, development and fitness of a polyphagous *Ceratitis* fruit fly, *Ceratitis fasciventris*, is adversely affected by host plant toxicity by comparing rates of development, survival and reproduction of captive bred individuals on four artificial media that differ in alkaloid concentration.

Chapter 1.2

It takes two to tango: the role of chemistry in evolutionary diversification of insect – host relationships

Abstract

Phytophagous insects and their host plants interact through diverse and complex relationships between host plant phenology, chemistry, selection, specialization, and detoxification and show related patterns of species diversification. Mutual interactions between phytophagous insects and plants provide little doubt that each group is in part responsible for the other's diversity, and plant-insect associations hence constitute an important component of ecosystems and the services they deliver. A better understanding of the processes underlying these interactions, such as plant phenology versus host selection by insects, or synthesis of secondary metabolites versus metabolic pathways to detoxify, will hence advance our understanding of ecosystems functioning. We here review different components of the co-evolutionary relationships between phytophagous insect and their host plants. We confer physiological and behavioral components that determine host plant choice and selection, discuss processes known to enhance host plant specialization and generalization, and pay special attention to chemical mediation of plant-insect relationships.

Ecology and evolution of insect-host associations

Over the last decades, studies on insect-plant interactions have shifted their focus from the ecology of plant-herbivore interactions to the ecology of multitrophic interactions (Dicke, 2000). Plants not only provide food, but they also affect phytophagous insects through, for example, physical structures and chemical information. Moreover, plants may interact in similar ways with pollinators and carnivorous insects, and this affects, or can be affected by, interactions with phytophagous insects depending on these plants (Dicke and Van Loon, 2000). Hereby, plants may provide an enemy-free or enemy-dense space to herbivores. Consequently, plant defense can be characterized as either direct or indirect. *Direct defense* is mediated by plant characteristics that affect the phytophages' biology, such as toxic secondary plant compounds. Secondary plant metabolites can considerably affect phytophagous insects by retarding their development, intoxicating them, or can be sequestered by herbivores insects (Coley and Barone, 1996). Which of these effects occur, depends on the specific metabolite-phytophagous combination. *Indirect defense*, in contrast, improves the performance of carnivorous insects, for example through the provision of shelter or infochemicals (Vet, 1999). Infochemicals are chemicals that convey interactions between organisms (Vet, 1999). They are divided into two major groups; pheromones, which are emitted and received by members of the same species and allelochemicals, emitted and received by members of different species (Noldus, 1989). Further, phenology of the host plant may be crucial for the performance of the insect, whereby disruption of the synchronization between insect and plant phenology may strongly influence insect population dynamics, for instance through climate change (Coley, 1998). Simultaneously, herbivory by phytophagous insects may exert a range of effects on plants such as altered shoot growth, root growth, flowering, and seed production, and shifts in plant chemistry and morphology (Crawley, 1997; Stanforth et al., 1997).

Ecological factors, such as described above, are thought to be selective agents that have led to the currently observed interactions between insects and plants. Several theories have been developed to explain these patterns. Overall, most of the current

hypotheses are based on two assumptions. The first is that attacks by phytophagous insects always reduce plant fitness. Therefore, insect attacks select for plant defense and/or escape mechanisms, such as secondary plant substances, mass fruiting, or changes in plant phenology. In essence, an evolutionary "arms race" is taking place between insects and plants. The second assumption is that insect-plant relationships are the result of unconstrained selection (Maynard Smith, 1990), posing that no species is so constrained that selection can not change it in any direction. This co-evolutionary theory suggests that insect-plant interactions arose through successive evolutionary innovations in plant defense and in insect attackers, thus producing alternating episodes of plant and insect radiation (Ehrlich and Raven, 1964). Such an evolutionary pattern would be expected to result in close similarity in the sequence of speciation events in the plants and insects (Thompson, 1994). These similarities, however, have been rarely observed and, consequently, have been assumed to play a minor role in the evolutionary associations between herbivores and their host-plants (Farrell and Mitter, 1993; Funk et al., 1995a, 1995b; Janz et al., 2001). In contrast, in the evolution of insect-plant relationships, plant chemistry has played an important role in insect host choice (Renwick and Chem, 1994), whereby host-plant shifts are usually constrained in chemical channels and represent colonization of pre-existing plant species that are phytochemically similar, but not necessarily taxonomically related, to their extant hosts (Feeny, 1992). Herbivore lineages may switch their affinities to other plant groups after diversification of the host-plants, with no induction of any adaptive response in these plants (Jermy, 1984, 1993). Indeed, based on observations of host-shifts over short periods of time, many authors have proposed that, on an evolutionary scale, host affiliations of herbivorous insects may not be a consistent feature (Bernays and Graham, 1988; Rausher, 2001). An example among the true fruit flies is the speciation of the *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae: Trypetinae) species complex (Bush, 1992). The documented switch of *R. pomonella* from native hawthorns, *Crataegus* spp., to the introduced apples in historical time led Bush (1969) to formulate a hypothesis of sympatric speciation via host shift. He suggested that the biological attributes of the insects, such as a positive correlation between host plant and mate selection and phenological differences between host races, permit new forms

to arise rapidly in the absence of geographical barriers to gene flow when host plant choice and diapause are under genetic control. Subsequently, *R. pomonella* host races on hawthorn and apple were documented based upon genetic (McPheron *et al.*, 1988), phenological (Smith, 1988), and behavioural (Prokopy *et al.*, 1988) differences evolving within the short time span after the host shift to apple. On the other hand, the European cherry fruit fly, *Rhagoletis cerasi* (L.), a species distantly related, but ecological similar to *R. pomonella* (Smith and Bush, 1997), occurs sympatrically on two different host plants, *Prunus avium* L. and *Lonicera xylosteum* L., and can be significantly distinguished in host populations due to geographical grouping (North and South), but did not establish a significant host race differentiation based upon genetic differences, resulting in minimal differences between host-associated populations of *R. cerasi* on *Lonicera* sp. and *Prunus* sp.

Host plant use: range, selection, find and accept

The width of the host plant niche occupied by a particular phytophagous insect can be constrained by one or several morphological, physiological and/or ecological factors, and it constitutes one of its main ecological characteristics. Insects may either exploit one single plant species (monophagy) or exploit different species belonging to one plant genus (stenophagy), one plant family (oligophagy) or several plant families (polyphagy) (White et al., 1999). Plant odor constitutes an important cue in host selection by phytophagous insects, and the strength of odor-induced attraction is assumed to be positively related to the degree of host specialization (Visser, 1986; Bernays and Chapman, 1994; Schoonhoven et al., 2005). Apart from olfactory sensors, visual stimuli are considered important as well (Bernays and Chapman, 1994). In this respect, Finch and Collier (2000) developed a theory based on "appropriate/inappropriate landings" to explain how pest insects of crucifers find their host. They suggest that insects can use visual stimuli to discriminate, for instance, between green plants and brown soil, but not between different plant species. Circumstantial evidence for the use of visual cues in host plants selection comes from

studies on chrysomelids where odor traps capture different numbers of insects depending on their colour (Láska et al., 1986; Hesler and Sutter, 1993; Szentesi et al., 2002; Yang et al., 2003). However, among *Phyllotreta* spp. (Chrysomelidae), where this phenomenon has been most frequently reported, wave length reflectances of the attractive traps did not match those of the host-plant leaves, suggesting that the visual cue may not be involved in host-plant selection (Yang et al., 2003). In *Rhagoletis pomonella* (Tephritidae), individuals were shown to detect odor of host fruit from over 20 m distance and visual stimuli of host trees from over 3 m distance (Green et al., 1994).

After location of a plant by a phytophagous insect, a second stage is its acceptance as a suitable host. The acceptance of a plant (as host) is said to occur when either sustained feeding or oviposition occurs. Acceptance is affected by motivation, the general willingness to feed or oviposit, which itself results from the integration of internal physiological state parameters (e.g. level of satisfaction, maturation state of eggs) of the insect. Host plant selection by ovipositing females is a key process critical for the survivorship, performance, and fitness of their offspring, especially in those species with low mobility of immature stages such as many Lepidopterans (Thompson and Pellmyr, 1991). Because plants have evolved a wide array of toxic chemical defences against phytophagous insects, the mechanism underlying host choice is expected to be highly related to the plant chemical composition (Honda, 1995; Jaenike, 1990). The ability to discriminate among plant chemical patterns varies depending on the degree of specialization (Janz and Nylin, 1997; Bernays, 2001; Egan and Funk, 2006; Wee and Singer, 2007). The Neural Limitation hypothesis predicts that, while generalists evaluate host suitability among different species with diverse chemistries, monophagous or oligophagous insects should have a greater capacity to differentiate host plant chemistry at the intraspecific level (Jaenike, 1990; Janz and Nylin, 1997). A narrow range of hosts, with reduced diversity of plant secondary chemicals, allows for greater efficiency in evaluating host suitability compared to generalists (Janz and Nylin, 1997; Bernays, 2001). Indeed, specialists are expected to select host plants and assess suitability by using a particular plant compound or mixtures of compounds that

function as reliable signals for host recognition (Feeny, 1992; Bernays, 2001). By being receptive to a set of high-contrast signals specific to their host plants that clearly stand out from non-host compounds, specialists might increase the speed and accuracy of host detection and appropriately evaluate host quality (Bernays, 2001).

Insect-host associations in tropical and temperate regions

Insect-plant interactions are believed to vary with latitude, due to differences in both insect and plant strategies and hence interactions, with more specialized phytophagous strategies (insects) and more efficient defence strategies (plants) occurring at lower latitudes (Coley and Barone, 1996; Grime, 2001; Pennings and Sillman, 2005). Some explanations for these differences assert that organisms in the tropics (at lower latitudes) have smaller niche sizes (MacArthur, 1969). For phytophagous insects, niche size is directly dependent on diet breadth, making information on host range critical to understanding the processes that lead to high diversity. The assumption of narrow host plant specializations among tropical herbivores insects was tested by Erwin (1982) who used the technique of canopy fogging to estimate insect diversity in a Panamanian rainforest. Results of that study, however, were strongly debated (Stork, 1988) and global trends in the degree of host plant specialization remain largely unresolved (Thomas, 1990). If tropical herbivores insects would show a higher degree of host plant specialization than taxonomically related temperate-zone species, this may (partly) explain the high species diversity of tropical biotas relative to those of temperate realms (but see Novotny and Basset, 2005). If, on the contrary, tropical herbivores would have roughly equal, or even broader, host plant ranges than their temperate-zone counterparts, then the hypothesis of narrow niche specializations as a mean of coexistence of highly speciose herbivore faunas would lose strength. Another aspect of host plant affiliations of herbivore insects is the well documented pose that vascular plants are, in general, much more diverse in tropical regions (Williams et al., 1994; Barthlott et al., 1996). Similarly, most higher taxa of insect herbivores exhibit a steep diversity gradient from the equator towards the polar regions (Rohde, 1992).

Floral diversity may hence be an important precondition for herbivore diversity. This was confirmed in a study comparing diet breadth among higher butterfly taxa between tropical and temperate-zone (Fiedler, 1998). Here, the results strongly challenged the view that tropical herbivores are generally more specialized than herbivores of higher latitudes. Rather, chemical constraints and phylogenetic conservatism shaped host plant associations in many taxa in such a way that differences between temperate and tropical representatives were slight. The results indicated that high floral diversity can be reflected by higher diversity of host plant affiliations of herbivores, but generalized conclusion should be scrutinized.

Processes underlying specialization on host plants

Host specialization is believed to constitute an important process underlying the exceptionally high level of diversification in insect species (Jaenike, 1990; Futuyama, 1991; Bernays and Chapman, 1994; Thompson, 1994). Niche breadth in host plant use has been shown to be constrained by morphological, physiological as well as ecological factors (Mattson et al., 1988), and insect taxa strongly vary in relative proportion of host specialists and host generalists. Various hypotheses have been invoked to explain this variation, such as the *physiological-efficiency* hypothesis (Janz et al., 2001), the *enemy-free space* (Stamp, 2001) hypothesis, the *optimal foraging* hypothesis (Scheirs, 2002), and the *neural-constraints* hypothesis (Bernays, 2001).

The *physiological-efficiency* hypothesis (Dethier 1954), states that herbivores adopt a specialist life-style by physiologically adapting to their host plants. While physiological adaptation of specialist species to the nutritional and chemical characteristics of their host plants has been shown in different studies (Appel and Martin, 1992; Barbosa et al, 1991; Berenbaum, 1981, 1983; Berenbaum et al, 1991), the prediction that specialist species perform better than generalists on host plants shared by both groups has only limited empirical support (Strauss and Zangerl, 2002). Also, there is weak or heterogenous support for two other predictions, i.e. that

utilization of different host-plants leads to physiological trade-offs (see comments by Strauss and Zangerl 2002) and that evolution of adult oviposition preference reflects that of immature performance (preference-performance correlation) (see comments by Scheirs et al. 2004).

The *enemy-free space* hypothesis (Jeffries and Lawton 1984) states that herbivores adopt a specialist life-style by using host plants as refuges against predators. Enemy-free space thus comprises defensive strategies such as the use of plant toxins for resistance to enemies (Singer and Stireman, 2005). This hypothesis has gained substantial empirical support and experiments showed that selection by generalist predators favours host specialists over generalists (Bernays, 1988; Bernays and Cornelius, 1989; Dyer, 1995, 1997; Vencl et al, 2005) while other studies showed that enemy-free space can be more important than food quality in determining host-plant preference (Damman, 1987; Denno et al, 1990; Baur and Rank, 1996; Camara, 1997a, 1997b, 1997c; Murphy, 2004).

The *optimal-foraging* hypothesis (Scheirs and De Bruyn, 2002) focuses on aspects of adult fitness such as mating success and realized fecundity. These aspects are likely to be influenced by life-history traits of insects, such as longevity, dispersal ability, and the use of host plants for mating sites, as well as by the availability of host plants. Phytophagous insects are expected to maximize their fitness by specializing on host plants on which females oviposit the maximum number of eggs (based on a combination or insect and plant traits), whereby the fitness contributions of adults are believed to outweigh those of immatures. Scheirs et al. (2000) addressed this issue by demonstrating the importance of adult fitness components in host use by grass-mining agromyzid flies.

The *neural-constraints* hypothesis (Bernays and Wcislo (1994) states that the limited ability of insects to process information, restricts their aptitude to make efficient decisions in finding and accepting potential host plants (Bernays, 1998). Inefficient decision making will be evolutionary penalized by reduced fitness from choosing

poor-quality food or poor oviposition sites. Because fitness costs and benefits are expressed in terms of food quality or realized fecundity costs, this hypothesis provides a mechanism underlying the other hypotheses discussed above.

Finally, also interspecific competition is generally believed to favour the evolution of ecological specialization, whereby in evolutionary time, competitors are predicted to evolve to exploit unoccupied niches (Abrams, 1987). For example, in the case of parasites, ecological competition between species is thought to promote the evolution of specialization to particular host species, allowing parasites to avoid competition or become better competitors (Poulin, 2007). However, in some cases competition may trade off against other factors to promote polyphagy. For example, polyphagy may be favored when food quality (in the absence of competitors) and competition intensity positively covary among different host plant species. Here, a phytophagous insect that competes poorly with others may gain a performance advantage by switching to alternative host plants under highly competitive circumstances, or may routinely use multiple host-plant species because highly competitive circumstances are unpredictable. In tephritid flies, interspecific competition commonly occurs (Fletcher, 1987). Numerous cases of species displacements attest for the occurrence of interspecific competition, particularly after invasions (Duyck *et al.*, 2004). After these tephritid invasions, also host plant shifts were observed (Duyck *et al.*, 2004). However, Duyck *et al.* (2008) observed no indication of niche partitioning for any of four abundant host fruits between four polyphagous fruit flies after invasion. The four fly species largely overlap in fruit exploitation; however, one species (*Ceratitis capitata*) was shown to be able to exploit rare fruit species that are not exploited by others present in the same climatic niche.

Chemical mediation of specialization on host plants

Secondary metabolites of plants trigger a wide range of effects in phytophagous insects, ranging from entirely harmless to highly toxic (Fraenkel, 1959), and hence affect their selection of host plants and level of ecological specialization. Because host plants are selected by adults yet mainly utilised by their larvae during feeding, coping with secondary metabolites involves behavioural as well as physiological mechanisms (Thompson, 1988). Based on the *preference-performance* hypothesis (Jaenike, 1978), ovipositing females should maximize their fitness by selecting plants in which offspring survival will be highest. However, empirical evidence for direct relationships between phytochemical defense in plants, chemically-mediated selection of host plant by phytophagous insects, and insect performance is scarce and restricted to a small number of taxa (Feeny, 1992). In butterflies of the family Papilonidae, the use of Rutaceae by the ancestor of all six species of the phytophagous *Papilio glaucus* group (North America) most likely resulted from a "key innovative" detoxification mechanism that allowed these species to exploit plant hosts that were previously resistant to hervivory (Berenbaum et al., 1996; Li et al., 2000). Less well understood, however, are the actual genes involved in the processing of chemical cues, despite the fact that successful infestation of previously "resistant" plants implicitly assumes that genetically-based variability in insect behavior, physiology, and/or ecology is involved (Scriber et al., 1991; Fritz and Simms, 1992; Coley and Barone, 1996; Caterino and Sperling, 1999). One superfamily of genes, the cytochrome P450 monooxygenases (P450s), is believed to participate in both biosynthetic and detoxification reactions and may therefore affect both host plant preference and insect performance (Mansuy, 1998). In *Papilio glaucus*, cytochrome P450 monooxygenase has been shown to be involved in the detoxification of furanocoumarins of Apiaceae and Rutaceae host plants. This gene family may also be involved in the metabolization of a wider range of toxins in host plants belonging to the Betulaceae, Magnoliaceae, and Rosaceae which are infested by two related *Papilio* species (*P. glaucus* and *P. canadensis*).

Conclusion

The evolutionary ecology of insect-host associations in phytophagous insect constitutes a versatile domain with many interactive components. Understanding insect-host interactions requires empirical and theoretical study of life-history traits of both plants and insects, such as plant phenology and host selection by insects, and synthesis of secondary metabolites by plants and metabolic pathways of insects to detoxify them. Such studies will provide a better insight in the ecological and evolutionary diversification of plants and of the insects that depend on them for feeding or reproduction. As insects have been shown to play a key role in diverse and complex ecological processes such as the course and rate of (host) plant succession (Whitham et al., 2006), studies of insect-plant interactions may ultimately advance our understanding of ecological and evolutionary processes underlying ecosystem functioning.

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Ceratitis capitata ovipositing © RS Copeland

Damage by Ceratitis cucurbitae © RS Copeland

Section II:

Spatial and temporal patterns in fruit fly host-use

Carpophthoromyia dimidiata male

Ceratitis cuthbertsoni female

Dacus sp. male

Trirhithrum demeyeri female

Capparimyia sp. on *Boscia sp.* (Capparaceae)

Bacterocera cucurbitae male

Representatives of six genera of the subfamily Dacinae © RS Copeland.

Chapter 2.1

Biogeography of ecological specialization in African fruit flies (Diptera: Tephritidae)

Abstract

Aim To examine the extent at which Tephritidae species richness, relative proportion of host specialists and generalists and level of endemism differ among 23 sub-biomes distributed across the African continent, Madagascar and offshore islands, and the Arabian Peninsula, and to explore relationships between patterns of species richness and host plant specialization.

Location Afrotropical region, Mascarenes, Madagascar and the Arabian Peninsula

Methods We obtained distributional and host plant data from six fruit infesting Tephritidae genera at sub-biome level from a relational database (http://projects.bebif.be/enbi/fruitfly). Based on these data, we established a species–area relationship and used chi-square tests to compare proportions of host plant specialists among genera and regions. Next, we calculated Sørensen indices to compare species composition across regions and performed a hierarchical cluster analysis to group sub-biomes based on their compositional similarity.

Results and discussion Species richness significantly varied among sub-biomes and increased with sub-biome area by a negative exponential function. Sub-biomes located near the equator boosted the highest species richness and highest proportion of stenophagous species. Similarity in species composition among regions was mainly explained by geographical proximity. At genus level, *Dacus* species were more abundant in dry areas, whereas *Ceratitis* species were more abundant in moist areas. Despite locally high levels of species richness, African fruit flies comprise few endemic species.

Introduction

One of the most striking patterns emerging from biodiversity studies is the disparity in species richness across taxa and geographic regions, as reflected by latitudinal gradients in species diversity from the poles to the equator with consistently higher species richness at lower latitudes (Rhode, 1992; Hillebrand, 2004). Biogeographical gradients such as these most likely result from shifts in the long-term balance between speciation and extinction rates (Wilson, 1992), possibly mediated by differential migration rates operating over shorter time-scales (Perry et al., 2009). The Resource-Use Hypothesis (Fernandez and Vrba, 2005) predicts higher levels of species richness and ecological specialization closer to the equator, due to higher past speciation rates in habitat specialists than in habitat generalists, and higher species richness in rainforests and deserts than in other ecosystems. It stresses the selection pressures associated with physical environmental changes as the direct promotors of vicariance (fragmentation of species' geographic distribution) and therefore of speciation: generalists are less susceptible to withdrawal of their resources, to strong directional selection and to vicariance as environments change. Thus generalists have lower speciation and extinction rates, whereas specialists are converse in all these respects. Since terrestrial biomes are characterized by gross vegetetional physiognomy, species that are restricted to a particular biome, or narrow range of vegetation physiognomy, are predicted to have high speciation rates in the face of recurrent environmental changes, while a species not restricted to a particular biome can use resources in more than one biome with predicted lower speciation rates. Alongside this latitudinal pattern in species richness, some authors claim that there is a general tendency for species richness to increase with the area of a biogeographical unit (May, 1975; Rosenzweig, 1995; Lomolino, 2000). This 'species-area relationship' (Lomolino, 2000) has been widely used as a criterion to identify areas with exceptionally high species richness, so-called biodiversity hotspots (Veech, 2000; Hobohm, 2003). However, the species-area relationship theory also has its critics following the development of neutral theory in macroecology, which provides atractable null hypothesis for community ecology, biogeography and conservation biology (Bell 2001, Hubbell 2001). The neutral theory

assumes there are no differences among individuals in terms of per capita vital rates or in their responses to the basic forces acting on a community. Making the neutrality assumption at the individual level rather than at the species level hence results in new explanations for the origin of a variety of macroecological patterns, including species richness and species-area relationships. However, this does not mean that area does not affect species diversity. One important mechanism underlying species-area relationships is the fact that habitat diversity tends to be positively related to habitat area, i.e. larger areas are predicted to host more species because they comprise a higher variety of habitats (Rosenzweig, 1995). Based on both ecological principles, large biomes located close to the equator are predicted to show high levels of species richness and high proportions of habitat specialists (Fernandez and Vrba, 2005).

Represented by an estimated three to ten million species (Ødegaard, 2006), insects constitute a significant component of the global biodiversity. Insects occupy a wide variety of (micro)habitats where they often play key functional roles (Kenis et al., 2009), and a vast majority display a phytophagous life-style (Strong et al, 1984). Phytophagous insects show diverse and complex relationships with their hostplants, and insights in insect-plant associations have significantly advanced our understanding of ecosystem functioning (Whitham et al., 2006). To better understand the evolutionary success of phytophagous insects, there is a need to study ecological factors that underlie their high level of ecological specialization, for instance in the choice of host plant for feeding or infestation (Ward et al., 2003; Morse and Farrell, 2005). As a first step to this end, we here document patterns in species richness and level of specialization on host plants in African fruit flies (Diptera: Tephritidae) at sub-biome level. Tephritidae are globally distributed picture-winged flies of variable size. With more than 4000 species described, they rank among the most diverse groups of true flies (White and Elson-Harris, 1992; Thompson, 1999). Tephritidae are predominantly phytophagous, with their larvae developing in the seed-bearing organs (flowerheads, fruits) of their host plants. Because a number of species infest economically important fruits (White and Elson-Harris, 1992), Tephritidae are regarded as important pest species (Thompson, 1999) and have hence been the subject

of intensive agricultural research. These true fruit feeding tephritids belong predominantly to the family Dacinae (White & Elson-Harris, 1992), sometimes classified as Dacini (Norrbom et al., 1999). Most of the species belong to two genera, *Ceratitis* (95 species) and *Dacus* (177 species) (White, 2006) while few others belong to closely- related genera such as the coffee fruit flies (*Trirhithrum* spp.) and the genus *Bactrocera*. Apart from their economic impact, fruit flies also constitute an interesting group to study ecological insect-host relationships. Most species are polyphagous, i.e. attack a wide variety of unrelated plant genera and families, while a smaller number is restricted to particular host families, genera or even single species of trees and shrubs. Many of these fruit flies are associated with forested areas, and might constitute good indicator species for the biodiversity of a given area. There are about 950 species and 150 genera of fruit fly (Tephritidae) known in Africa (Crosskey, 1980), most of which form a natural component of Africa's rich and varied biodiversity.

Here we study to what extent, and in what direction, species richness, level of endemism and relative proportion of specialist and generalist Tephritidae differ among 23 sub-biomes distributed across the Afrotropical region, including the African continent south of the Sahara, Madagascar and offshore islands, and part of the Arabian Peninsula (see Crosskey, 1980 for full description). Sub-biomes are defined as biogeographic areas with climates, landscapes, and biotic assemblages that are more similar to each other than to those in adjacent regions (e.g. Ricketts et al., 1999). Sub-biomes can be clustered into seven conceptual biomes at a continental scale (Whittaker *et al.*, 1975; Ricklefs and Miller, 1999). While this paper mainly focuses on biogeographic patterns at sub-biome level, we also compare moist and dry biomes. Analyses are based on distributional data of 344 native fruit infesting *Bactrocera, Ceratitis, Dacus, Carpophthoromyia, Trirhithrum* and *Capparimyia*. Relationships between sub-biome area and species richness are assessed by species-area curves, while similarities in species diversity among sub-biomes are compared by Sørensen similarity indices and cluster analysis. We compare relative distributions of host plant specialists and generalists between two genera, *Ceratitis* and *Dacus*, for which detailed

data are available. We test the Resource-Use Hypothesis by exploring possible relationships between patterns of species richness and host plant specialization.

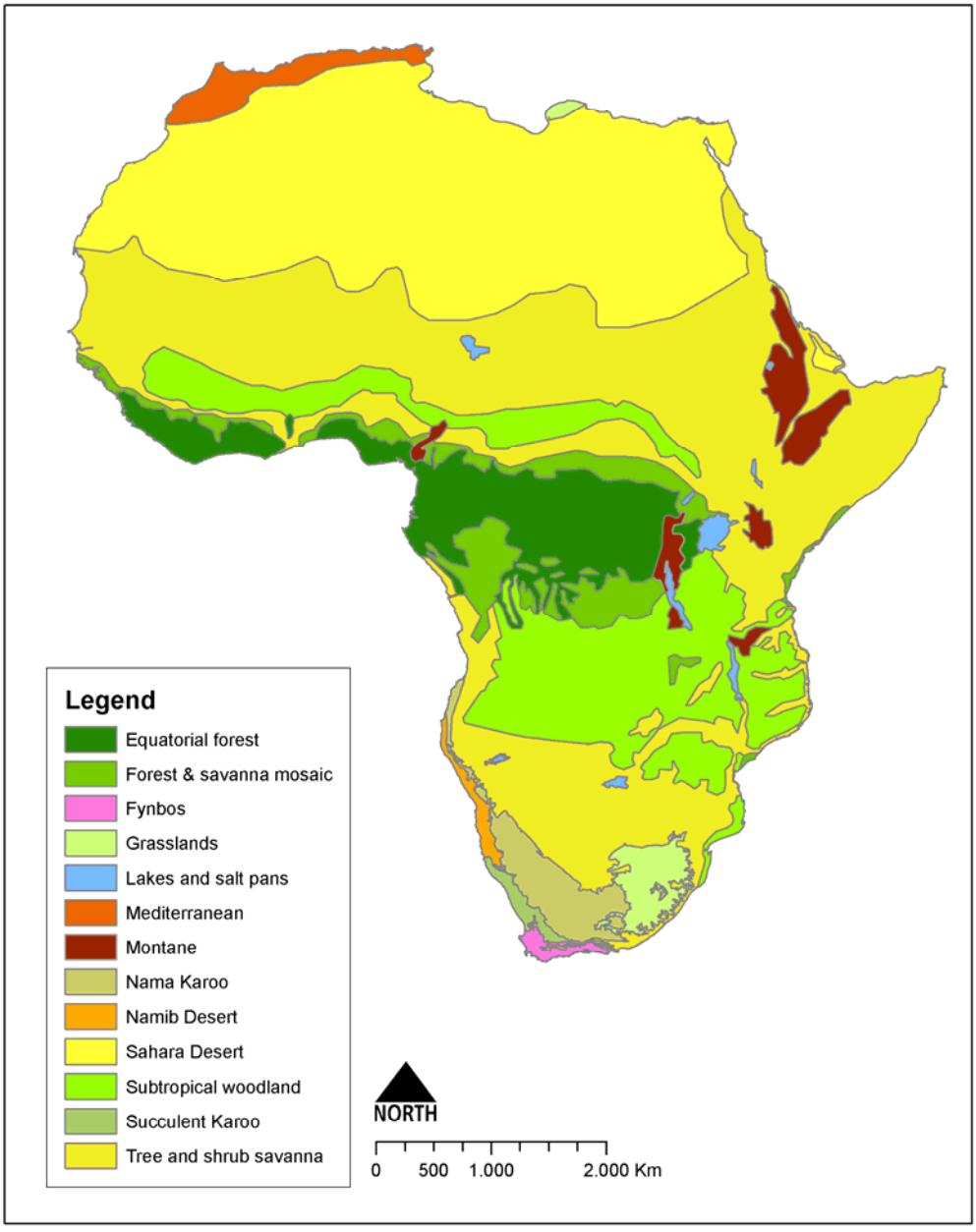

Figure 1 Biomes of Africa

Materials and Methods

Study species

Site locations and host plant use of 5151 block records (whereby a block record represents all sampling events at a particular location) comprising about 87000 individuals from 344 Tephritidae species were obtained from a relational database managed at the Royal Museum for Central Africa (http://projects.bebif.be/enbi/fruitfly; see Appendix 1). Site records comprise the Afrotropical region, Madagascar and the Mascarenes included, and the southern part of the Arabian Peninsula (see Burgess et al., 2006). There were no specimens included from the genera *Perilampsis* Bezzi or *Neoceratitis* Hendel since no reliable recent revisions were available. After excluding non-indigenous species known to have invaded the African region (e.g. several Asian *Bactrocera* species; White, 2006), data from six frugivorous genera of the subfamily Dacinae (*Bactrocera, Ceratitis, Dacus, Carpophthoromyia, Trirhithrum* and *Capparimyia*) indigenous to the study region were retained for further analysis. *Bacterocera* Macquart and *Dacus* Fabricius are genera belonging to the tribe Dacini (Dacinae) of which species are concentrated in two areas of the world (Drew *et al.*, 1998). The two areas are the Afrotropical region and from Southeast Asia to Northeeastern Australia. The genera *Ceratitis* McLeay, *Capparimyia* Bezzi, *Trirhithrum* Bezzi and *Carpophthoromyia* Austen belong to the tribe Ceratidini (Dacinae) with Afrotropical occurance (De Meyer, 1999 and references herein, 2006; De Meyer and Freidberg, 2005). The study area comprises eight biogeographic biomes which are subdivided into 23 sub-biomes and 52 ecoregions (Burgess et al 2004; Table 1). Data on plant richness at sub-biome level were obtained from Burgess et al (2004) and Kier et al (2005).

Species richness and host plant use

Species richness *S* was calculated as the number of different species per sub-biome (Table 1). To model species-area relationships at sub-biome level, we transformed *S* by applying a power function $S = aA^b$ (Veech, 2000; Fattorini, 2006) with *a* and *b* constant for a particular group of species distributed among a particular set of sub-biomes (details in Tjørve, 2003, 2009). A negative exponential distribution curve $S=a(1-\exp(-bA))$ best fitted our data. To study distribution patterns in host plant use, each fruit fly species was subsequently assigned to one of two host use categories (*stenophagous*, i.e. infesting one or several species of one host plant genus; or *polyphagous*, i.e. infesting several species of one or more host plant families; White et al., (2000)) and the proportion of stenophagous (specialist) and polyphagous (generalist) species per sub-biome was calculated.

Species similarity indices

We calculated Sørensen incidence-based similarity indices (Chao *et al.*, 2005) between each pair of sub-biomes and used a hierarchical average linkage cluster analysis (Van Tongeren, 1995) to group sub-biomes based on their compositional similarity in Tephritidae. Number of shared species and Sørensen's indices were calculated with EstimateS Vs6 (Colwell, 2001) while cluster analysis was performed with Systat Vs 8.0 (Systat-Products, 1998). To avoid false negatives, cluster analysis was restricted to species for which a minimum of 23 block records (i.e. number of different sub-biomes) was available, whereby a block record represents all sampling events at a particular location. Species for which all block records (minimum of 23 records) were restricted to one single sub-biome were considered endemic to this sub-biome.

Results

Species richness and distribution

Sub-biomes significantly varied in level of species richness, with the highest richness recorded in Afromontane Forest and the lowest richness in Island Dry Forest (Table 1). A negative exponential distribution curve $S=a(1\text{-}\exp(-bA))$ with a= 128.28 and b= 1.2502×10^{-6} best described the relationship between species richness and area of a given sub-biome, reaching an asymptotic value from 4.000.000 km^2 onwards (Fig. 1). Afromontane Forest and Acacia Savanna Woodland contained more species, and Southern African Xeric Woodland-Shrubland and Miombo Woodland contained less species than expected from their respective areas (Fig. 2). Sub-biomes that contained the highest number of Tephritidae species also showed the highest level of plant richness (Table 1). Sørensen coefficients and two-way cluster analysis of similarities in species composition per sub-biome (Figure 4), revealed strong mutual species overlap between Afromontane Forest (SB1), Acacia Savanna Woodland (SB8), Miombo Woodland (SB11), Montane-Forest Grassland Mosaic (SB18), between Guineo-Congolian Lowland Moist Forest (SB3), and Guineo-Congolian Forest Savanna Mosaic (SB9) and between Mopane Woodland (SB12) and East African Mangroves (SB25) (Table 1). None of the species were recorded in all sub-biomes, but most species were present in sub-biomes SB1, SB8, SB11, SB18, SB3 and SB9 (Fig. 4). A total of 31 species were endemic at sub-biome level (distributed over 11 sub-biomes) (Table 1), with Island Moist Forest and Guineo-Congolian Lowland Moist Forest hosting the highest number of endemics (Table 1), representing all genera except *Capparimyia*. At biome-level, species richness was highest in Tropical And Subtropical Moist Broadleaf Forest and lowest in Tropical And Subtropical Dry And Broadleaf Forest (Table 1).

Distribution of host plant specialists and generalists

Among the 344 Tephritidae species recorded, 108 species were stenophagous, 74 species were polyphagous, while for 162 other species host use remained unknown (Appendix 1). Sub-biomes with lower species richness (i.e. located under the SAR-curve) hosted a significantly smaller proportion of stenophagous species (χ_1^2 =15.7; p<0.0001) while species-rich sub-biomes (i.e. located above the SAR-curve) showed an opposite trend, albeit not statistically significant (χ_1^2=3.4; p=0.06) (Fig. 3). The relative proportion of stenophagous and polyphagous species significantly differed between sub-biomes positioned above (SB1, SB8) or below (SB11, SB24) the species-area curve (χ_1^2=9.2; p=0.003): the former showed equal proportions of stenophagous and polyphagous species (50 stenophagous: 50 polyphagous), whereas stenophagous species were significantly less represented in the latter (30 stenophagous: 70 polyphagous). Cluster analysis identified three groups of polyphagous species: (i) *Ceratitis rosa*, *Dacus durbanensis*, *Carpophthoromyia dimidiata*, *Dacus lounsburyii*, *Dacus mulgens*; (ii) *Dacus ciliatus*, *Capparimyia bipustulata*; (iii) *Ceratitis anonae*, *Trirhithrium coffeae*, that were particularly abundant in sub-biomes SB1, SB8, SB11, SB18, SB3 and SB9 (Fig. 4). Species of cluster (i) were also abundant in sub-biomes SB2, SB12 and SB25 but absent from SB3 and SB9. Stenophagous species did not significantly cluster (Fig. 4). At biome level, the ratio of stenophagous to polyphagous species differed significantly between Biomes 1 (Tropical And Subtropical Moist Broadleaf Forest) and 13 (Desert) (χ_1^2= 21,3 p<0.001) with a higher proportion of stenophagous species in the former and of polyphagous species in the latter.

Distribution at genus level

The proportion of species belonging to the genera *Ceratitis* and *Dacus* differed significantly among sub-biomes (χ_1^2= 220.9; p<0.0001). At biome level, the proportion of *Ceratitis* species was significantly higher than of *Dacus* species in Biome 1 (Tropical And Subtropical Moist Broadleaf Forest), whereas the opposite pattern

occurred in Biome 13 (Desert And Xeric Scrublands) (χ_1^2= 6.1; p=0.01) The relative proportion of stenophagous and polyphagous species did not differ between both genera (χ_1^2= 0.006: p=0.9). At genus level, stenophagous nor polyphagous species significantly clustered together in sub-biomes (Figure 4).

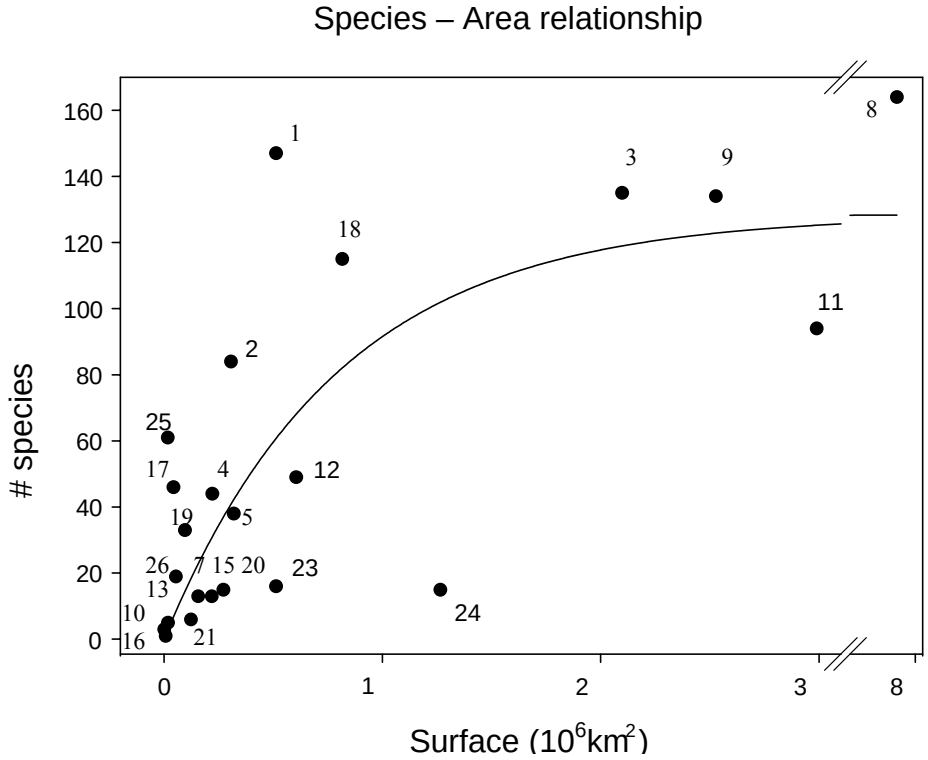

Figure 2.
Relationship between species richness per sub-biome and its surface area. SAR equation modelled as $S=128.28A^{1.2\ 10\text{-}6}$. Numbers indicate sub-biomes according to Burgess et al.(2004); 1: Afromontane forest; 2: Guineo-Congolian lowland moist forest; 3: Guineo-Congolian lowland moist forest; 5: Island moist forest; 8: Acacia savanna woodland; 9: Guineo-Congolian forest savanna mosaic; 11: Miombo woodland; 12: Mopane woodland; 13: Seasonal grassland; 15: Freshwater wetland; 17: Alpine moorland; 18: Montane forest-grassland mosaic; 19: Mediterranean scrub; 20: Desert; 21: Island xeric woodland-shrubland Madagascar; 23: Northeastern African xeric woodland-shrubland; 24: Southern African xeric woodland-shrubland; 25: East African mangroves; 26: West African mangroves.

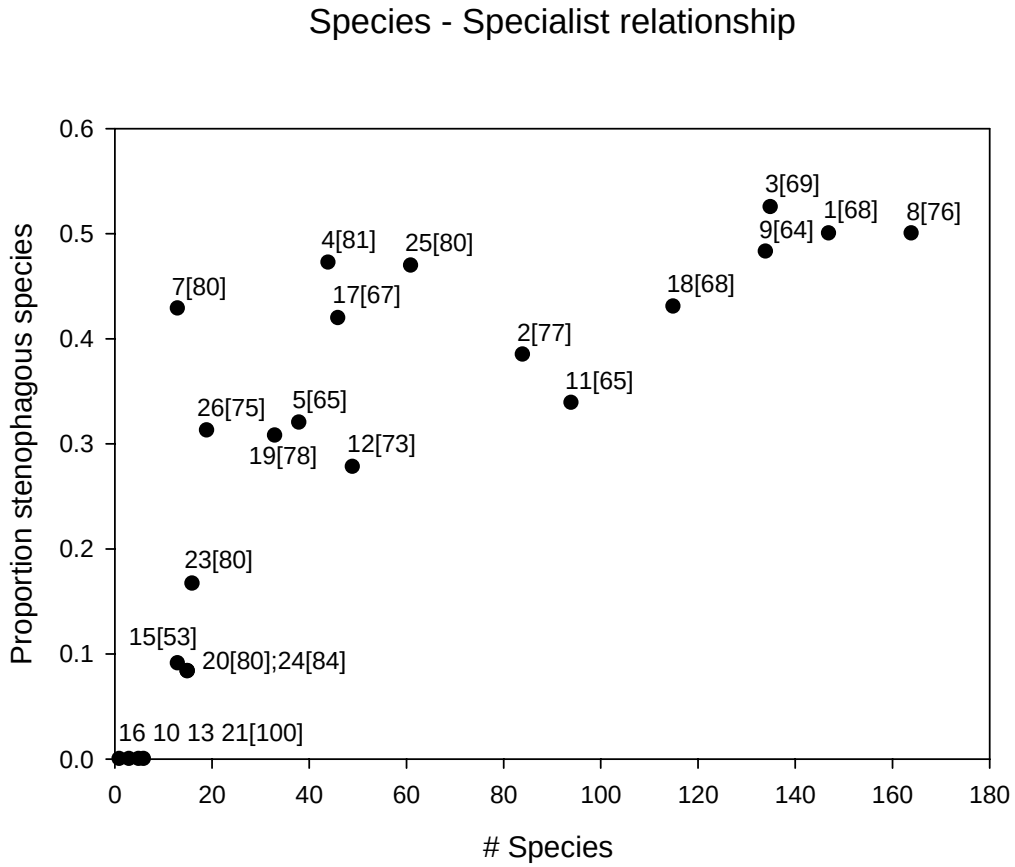

Figure 3.
Comparison of the proportion of stenophagous species vs species richness between sub-biomes. Numbers indicate sub-biomes according to Burgess et al.(2004); Numbers between brackets indicate the percentage of species of which the host-use strategy is known. 1: Afromontane forest; 2: Guineo-Congolian lowland moist forest; 3: Guineo-Congolian lowland moist forest; 5: Island moist forest; 8: Acacia savanna woodland; 9: Guineo-Congolian forest savanna mosaic; 11: Miombo woodland; 12: Mopane woodland; 13: Seasonal grassland; 15: Freshwater wetland; 17: Alpine moorland; 18: Montane forest-grassland mosaic; 19: Mediterranean scrub; 20: Desert; 21: Island xeric woodland-shrubland Madagascar; 23: Northeastern African xeric woodland-shrubland; 24: Southern African xeric woodland-shrubland; 25: East African mangroves; 26: West African mangroves.

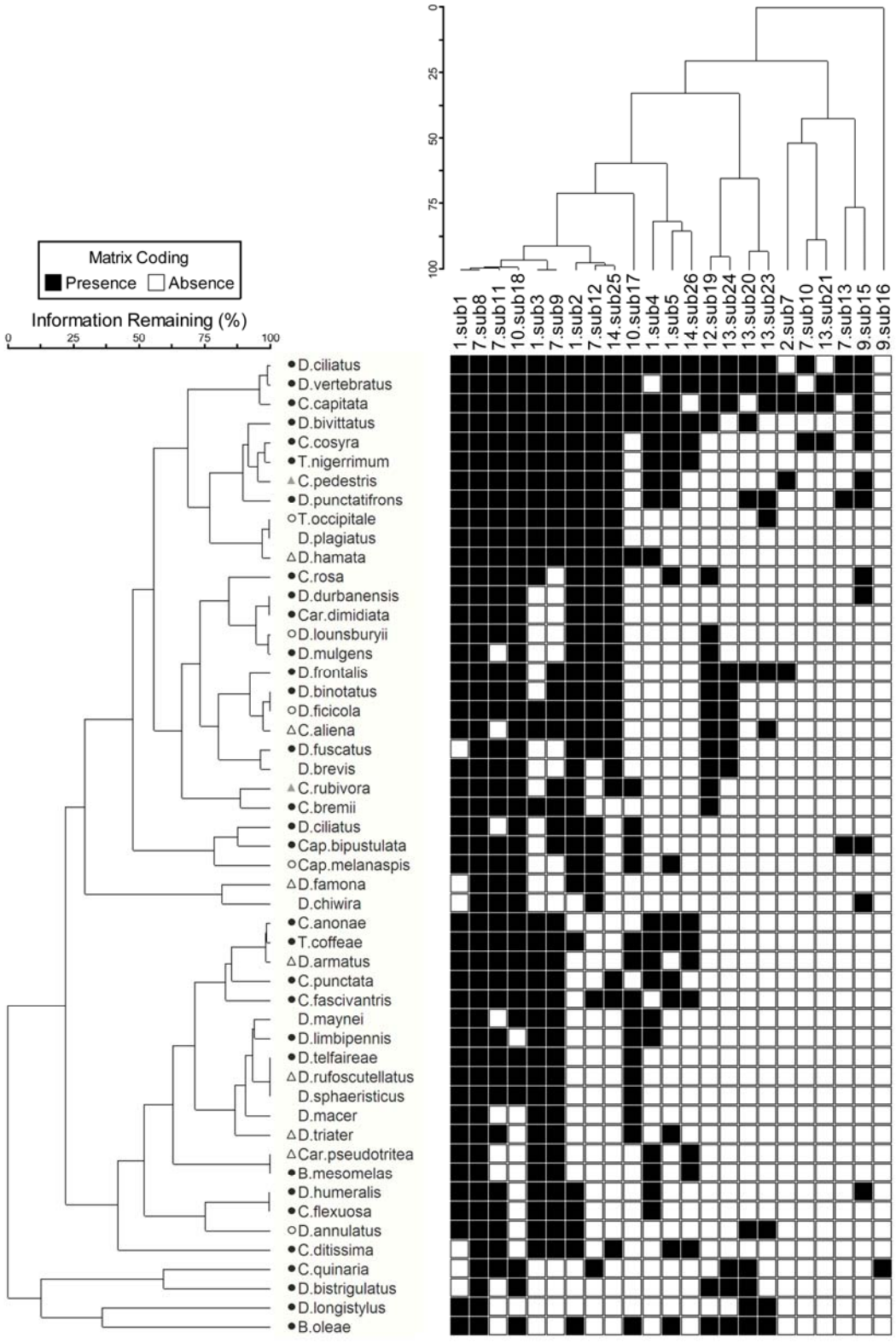

Figure 4.
Cluster diagram grouping sub-biomes based on their compositional similarity calculated by Sørensen incidence-based similarity indices. The Sørensen index is a measure of similarity between two sub-biomes, based on the number of shared species (*D: Dacus, C: Ceratitis, T: Trirhithrum, Cap: Carpophthoromyia*. Host-use preferences are indicated per species: Δ host range 1 species; ▲ host range 1 genus; ○ host range 1 family; ● host range > 1 family. For sub-biome names see Fig. 1 and 2.

Table 1.
List of biomes and sub-biomes with indication of fruit fly richness (biome and sub-biome), number of fruit fly endemics (sub-biome), plant richness (sub-biome) and geographic area (sub-biome).

Biome	***Sub-biome***	***fruit fly Species Richness***	***Fruit Fly Endemics***	***Plant Species Richness***	***Area in km²***
1.tropical and subtropical moist broadleaf forests 270spp.	1. Afromontane forest	147	2	24900	512898
	3. Guineo-Congolian lowland moist forest	135	7	29200	2097593
	2. Eastern African lowland forest-grassland mosaic	84	2	7700	306539
	4. Guineo-Congolian swamp forest	44	0	4400	220375
	5. Island moist forest	38	9	11930	319063
7.tropical and subtropical grasslands, savannas, shrublands and woodlands 239spp.	8. Acacia savanna woodland	164	4	15000	7745652
	9. Guineo-Congolian forest savanna mosaic	134	2	14600	2527125
	11. Miombo woodland	94	1	12500	2989314
	12. Mopane woodland	49	0	3600	604902
	13. Seasonal grassland	5	0	1200	17948
10.montane grasslands 136spp.	18. Montane forest-grassland mosaic	115	1	19900	815798
	17. Alpine moorland	46	0	2300	42877
14. mangroves 72spp.	25. East African mangroves	61	1	250	17006
	26. West African mangroves	19	0	250	54214
12. Mediterranean forsests, woodlands and scrub 33spp.	19. Mediterranean scrub	33	1	10700	95608
13. deserts and xeric shrublands 33spp.	23. Northeastern African xeric woodland-shrubland	16	0	4250	512914
	20. desert	15	0	1800	271260
	24. Southern African xeric woodland-shrubland	15	0	7750	1265060
	21. Island xeric woodland-shrubland Madagascar	6	0	2680	122949
	10. Island xeric woodland-shrubland St. Helena	3	0	x	130
9. flooded grasslands and savannas 14spp	15. Freshwater wetland	13	0	3000	218230
	16. Saline wetland	1	0	80	7208
2. tropical and subtropical dry and broadleaf forests 13sp	7. Island dry forest	13	1	1200	156112

Discussion

Our study reveals significant variation in richness of tephritid fruit flies among different African sub-biomes, with the highest levels recorded in sub-biomes located closest to the equator, i.e. Afromontane Forest, Acacia Savanna Woodland, Guineo-Congolian Lowland Moist Forest, Guineo-Congolian Forest Savanna Mosaic and Montane Forest-Grassland Mosaic. Across all sub-biomes, larger entities hosted more fruit fly species than smaller ones, supporting the universal species-area relationship confirmed for a wide variety of plant and animal taxa (e.g. MacArthur and Wilson, 1967). Some sub-biomes showed higher or lower levels of species richness than predicted from their respective area, however, supporting the notion that species richness may not be linked to area per se, but rather to drivers that are indirectly related to area, such as habitat heterogeneity (Rosenzweig 1995; Whittaker 1998; Tews *et al.* 2004).

Among the most species-rich equatorial sub-biomes, the Afrotropical Forest sub-biome, in particular, is known for its high complexity in vegetation composition and structure (Burgess *et al.,* 2004). Within this sub-biome, biodiversity hotspots such as the Eastern Arc Mountains and coastal forests of Tanzania and Kenya (Eastern Arc) show exceptionally high richness of (endemic) plants and animals (Myers, *et al.*, 2000; Lovett *et al.,* 2005; Burgess *et al.,* 2007) which can be expected to comprise a high variety in suitable host plants for fruit-infesting Tepritidae. In contrast, Southern African Xeric Woodland-Shrubland and Miombo Woodland showed lower levels of species richness than expected from their respective areas. Both sub-biomes are highly homogenous in structure (Burgess *et al.*, 2004), and absence of a positive relationship between habitat area and habitat heterogeneity has earlier been shown to reverse the sign of the species-area relationship (Baldi 2008). On the other hand, the Mediterranean Scrub sub-biome did not host a high number of fruit fly species, despite the high plant species richness in the Karoo region (Burgess et al., 2004). Despite the high species richness in the Afrotropical Forest sub-biome, only two species (*Dacus basifasciatus* and *Dacus ruslan*; host ranges unknown) are endemic to this sub-biome.

Because host plants are not necessarily restricted to a single biogeographical unit, the probability that suitable host species occur in more than one sub-biome is high, especially for polyphagous species that may infest up to twenty different hosts (De Meyer, 2001). The high number of endemic Tephritidae recorded in the Island Moist Forest sub-biome, despite its moderate level of species richness, may therefore more likely be related to the high degree of isolation of oceanic islands than to their level of habitat heterogeneity (Gruner, 2007).

As predicted by the Resourse Use hypothesis (Fernandez and Vrba, 2005), the proportion of host specialists (stenophages) within Tephritidae communities was highest in species-rich sub-biomes located close to the equator. Most of these sub-biomes are part of the Tropical And Subtropical Moist Broadleaf Forest (TSMBF) biome, and species from rainforest sub-biomes are believed to have undergone higher speciation rates, in particular habitat specialist species (Fernandez and Vrba, 2005). In contrast, species poor sub-biomes away from the equator comprised proportionally more generalist (polyphagous) species. Contrary to the prediction from the Resource Use hypothesis, however, the Desert biome comprised only few stenophagous species. When comparing Tephritidae communities between the TSMBF and Desert biomes at genus level, *Ceratitis* species dominated the former biome whereas *Dacus* species dominated the latter. Dominaton of *Dacus* species in dryer habitats may be related to their particular host use range, which is confounded to Asclepiaceae, Passifloraceae and Curcubitaceae (Virgilio *et al.*, 2009). These plant families predominantly consist of creepers which reach their highest abundances in dryer areas, probably because their fruits are better adapted to cope with dryness (Heywood, 1998). Fruit fly species of the genus *Ceratitis*, in contrast, dominantly infest fleshy fruits from trees ((http://projects.bebif.be/enbi/fruitfly) which are more abundant in forests. Molecular (Virgilio *et al.*, 2009; Barr and McPheron, 2006; ms 3) and morphological data (De Meyer, 2005) earlier revealed different monophyletic clades of stenophagous species in the genus *Ceratitis*, all members of which inhabit the TEMBF biome. In contrast, only one such clade was apparent in the genus *Dacus*, all three members of which also inhabit the TEMBF biome (and none in the Desert biome) (Virgilio *et al.*, 2009).

Analysis of species composition showed strong similarities in Tephritidae communities among species-rich sub-biomes, whereas species-poor ones mainly contained subsets of the latter (nested subset structure sensu Worthen, 1996). Clustering of sub-biomes based on their richness of Tephritidae species did not coincide with the classification of sub-biomes in biomes following Burgess et al. (2006). Since ecological factors underlying patterns of species composition are believed to drive patterns in species richness too (Lomolino 2000), higher similarities in Tephritidae communities can be predicted among geographically neighbouring sub-biomes that are more prone to inter-biome migration (Nekola and White, 1999; Ricklefs and Lovette, 1999; Azeria, 2004). Results of this study largely support this prediction. For example, the East African and West African Mangrove sub-biomes did not cluster together, likely because the former is more closely located to the species-rich Eastern Arc and East African Coastal Forest biodiversity hotspot from which it contains a nested species subset, with the exception of two species, *Dacus fuscatus* and *Ceratitis ditissima*. However, West African Mangrove sub-biome also contains a species subset, similar to the East African mangroves, but smaller. Likewise, Northeastern African Xeric Woodland-Shrubland and Southern African Xeric Woodland-Shrubland sub-biomes did not cluster together (similarities in Tephritidae communities are mainly driven by widespread *Dacus* species that have their main distribution in dry areas), but the former clustered with the more nearby Desert sub-biome. Other examples involved the non-clustering of the Island Xeric Woodland-Shrubland sub-biome with the other desert sub-biomes (probably due to the isolation effect described above) and the clustering of the Mediterranean Scrub and Southern African Xeric Woodland-Shrubland sub-biomes (mainly South Africa) and the Seasonal Grassland and Freshwater Wetland sub-biomes (mainly Tanzania).

Conclusion

Results of this study reveal significant biogeographic patterns in the distribution of Tephritidae species across the African continent, Madagascar and offshore islands, and the Arabian Peninsula. Overall, species richness increases with sub-biome area and reaches its highest levels near the equator. Equatorial sub-biomes also contain the highest proportion of stenophagous species, supporting the *resource-use* hypothesis that predicts positive relationships between species richness and ecological specialization. Similarity in species composition among biogeographic regions is mainly explained by geographical proximity. Between genera, there was no difference in specialist to generalist ratio. However, representation of the genera *Ceratitis* and *Dacus* differed between regions of the tropical and subtropical moist broadleaf forest and of the desert and xeric scrublands, whereby *Dacus* species were more abundant in dry areas and *Ceratitis* species in moist areas. Despite high levels of species richness in equatorial sub-biomes, Tephritidae comprise remarkably few endemic species.

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Appendix 1
List of fruit fly species included
S: Stenophagous; P: Polyphagous;
X: Status unknown

Bactrocera.amplexa	S
Bactrocera.montyanus	S
Bactrocera.nigrivenata	S
Capparimyia.maeruae	S
Capparimyia.mirabilis	S
Capparimyia.spatulata	S
Carpophthoromyia.interrupta	S
Carpophthoromyia.litterata	S
Carpophthoromyia.pseudotritea	S
Carpophthoromyia.scutellata	S
Carpophthoromyia.tritea	S
Carpophthoromyia.vittata	S
Trirhithrum.albonigrum	S
Trirhithrum.argenteocuneatum	S
Trirhithrum.demeyeri	S
Trirhithrum.dimorphum	S
Trirhithrum.divisum	S
Trirhithrum.leonense	S
Trirhithrum.manganum	S
Trirhithrum.nigrum	S
Trirhithrum.notandum	S
Trirhithrum.obscurum	S
Trirhithrum.overlaeti	S
Trirhithrum.psychotriae	S
Trirhithrum.stecki	S
Trirhithrum.teres	S
Trirhithrum.validum	S
Ceratitis.acicularis	S
Ceratitis.aliena	S
Ceratitis.antistictica	S
Ceratitis.argenteostriata	S
Ceratitis.bicincta	S
Ceratitis.brachychaeta	S
Ceratitis.connexa	S
Ceratitis.divaricata	S
Ceratitis.ealensis	S
Ceratitis.flava	S
Ceratitis.fulicoides	S
Ceratitis.gravinotata	S
Ceratitis.lepida	S
Ceratitis.malgassa	S
Ceratitis.millicentae	S
Ceratitis.mlimaensis	S
Ceratitis.munroi	S
Ceratitis.nigricornis	S
Ceratitis.obtusicuspis	S
Ceratitis.oraria	S
Ceratitis.paracolae	S
Ceratitis.pennitibialis	S
Ceratitis.perisae	S
Ceratitis.pinax	S
Ceratitis.roubaudi	S
Ceratitis.serrata	S
Ceratitis.stictica	S
Ceratitis.tananarivana	S
Ceratitis.tripteris	S
Ceratitis.turneri	S
Ceratitis.whitei	S
Dacus.adenionis	S
Dacus.adustus	S
Dacus.apostata	S
Dacus.arcuatus	S
Dacus.armatus	S
Dacus.aspilus	S
Dacus.botianus	S
Dacus.brevistriga	S
Dacus.ceropegiae	S
Dacus.chrysomphalus	S
Dacus.cyathus	S
Dacus.diastatus	S
Dacus.disjunctus	S
Dacus.eclipsis	S
Dacus.famona	S
Dacus.gabonensis	S
Dacus.hamatus	S
Dacus.inflatus	S
Dacus.langi	S
Dacus.masaicus	S
Dacus.mediovittatus	S
Dacus.obesus	S
Dacus.okumuae	S
Dacus.pergulariae	S
Dacus.phloginus	S
Dacus.purus	S
Dacus.rufoscutellatus	S
Dacus.rufus	S
Dacus.theophrastus	S
Dacus.transitorius	S
Dacus.triater	S
Dacus.umbrilatus	S
Dacus.viator	S
Dacus.yangambinus	S
Trirhithrum.culcasiae	S
Trirhithrum.inscriptum	S
Ceratitis.argenteobrunnea	S
Ceratitis.cornuta	S

Ceratitis.curvata	S
Ceratitis.munroanum	S
Ceratitis.scaevolae	S
Dacus.apoxanthus	S
Dacus.venetatus	S
Capparimyia.savastani	S
Trirhithrum.albomaculatum	S
Ceratitis.marriotti	S
Ceratitis.simi	S
Ceratitis.lobata	S
Ceratitis.pedestris	S
Ceratitis.rubivora	S
Trirhithrum.nitidum	P
Ceratitis.perseus	P
Dacus.annulatus	P
Dacus.eminus	P
Dacus.ficicola	P
Dacus.inornatus	P
Dacus.pullescens	P
Dacus.sphaeristicus	P
Bactrocera.mesomelas	P
Carpophthoromyia.dividua	P
Trirhithrum.fraternum	P
Ceratitis.lentigera	P
Ceratitis.semipunctata	P
Ceratitis.silvestrii	P
Dacus.adenae	P
Dacus.durbanensis	P
Dacus.longistylus	P
Ceratitis.podocarpi	P
Dacus.umbeluzinus	P
Bactrocera.oleae	P
Ceratitis.contramedia	P
Ceratitis.cosyra	P
Ceratitis.cuthbertsoni	P
Ceratitis.penicillata	P
Ceratitis.venusta	P
Dacus.lounsburyii	P
Ceratitis.hamata	P
Dacus.briani	P
Carpophthoromyia.dimidiata	P
Ceratitis.discussa	P
Ceratitis.morstatti	P
Ceratitis.quinaria	P
Dacus.humeralis	P
Ceratitis.colae	P
Ceratitis.flexuosa	P
Dacus.binotatus	P
Ceratitis.bremii	P
Capparimyia.aenigma	P
Capparimyia.bipustulata	P
Dacus.limbipennis	P
Ceratitis.striatella	P
Dacus.bistrigulatus	P
Dacus.ostiofaciens	P
Dacus.telfaireae	P
Bactrocera.biguttula	P
Dacus.mulgens	P
Dacus.tenebricus	P
Ceratitis.edwardsi	P
Dacus.siliqualactis	P
Ceratitis.querita	P
Ceratitis.stipula	P
Capparimyia.melanaspis	P
Dacus.frontalis	P
Ceratitis.punctata	P
Trirhithrum.occipitale	P
Dacus.vertebratus	P
Trirhithrum.coffeae	P
Ceratitis.copelandi	P
Ceratitis.cristata	P
Trirhithrum.meladiscum	P
Dacus.fuscatus	P
Ceratitis.ditissima	P
Ceratitis.caetrata	P
Dacus.demmerezi	P
Trirhithrum.senex	P
Dacus.bivittatus	P
Ceratitis.catoirii	P
Dacus.punctatifrons	P
Dacus.ciliatus	P
Trirhithrum.nigerrimum	P
Ceratitis.anonae	P
Ceratitis.fasciventris	P
Ceratitis.capitata	P
Ceratitis.rosa	P
Bactrocera.cogani	X
Bactrocera.lucida	X
Bactrocera.menanus	X
Bactrocera.munroi	X
Bactrocera.nesiotes	X
Capparimyia.aristata	X
Carpophthoromyia.debeckeri	X
Carpophthoromyia.flavofasciata	X
Carpophthoromyia.nigribasis	X
Carpophthoromyia.procera	X
Carpophthoromyia.radulata	X
Carpophthoromyia.speciosa	X
Carpophthoromyia.tessmanni	X
Carpophthoromyia.virgata	X
Trirhithrum.albopleurale	X
Trirhithrum.basale	X
Trirhithrum.bimaculatum	X
Trirhithrum.brachypterum	X
Trirhithrum.crescentis	X
Trirhithrum.homogeneum	X
Trirhithrum.iridescens	X
Trirhithrum.micans	X

Trirhithrum.ochriceps	X	Dacus.delicatus	X
Trirhithrum.quadrimaculatum	X	Dacus.deltatus	X
Trirhithrum.resplendens	X	Dacus.elatus	X
Trirhithrum.scintillans	X	Dacus.elegans	X
Trirhithrum.stubbsi	X	Dacus.elutissimus	X
Trirhithrum.transiens	X	Dacus.erythraeus	X
Trirhithrum.viride	X	Dacus.etiennellus	X
Ceratitis.andranotobaka	X	Dacus.externellus	X
Ceratitis.barbata	X	Dacus.fasciolatus	X
Ceratitis.chirinda	X	Dacus.fissuratus	X
Ceratitis.dumeti	X	Dacus.flavicrus	X
Ceratitis.epixantha	X	Dacus.freidbergi	X
Ceratitis.faceta	X	Dacus.fumosus	X
Ceratitis.grahami	X	Dacus.fuscinervis	X
Ceratitis.guttiformis	X	Dacus.fuscovitttatus	X
Ceratitis.hancocki	X	Dacus.ghesquierei	X
Ceratitis.inauratipes	X	Dacus.guineensis	X
Ceratitis.lineata	X	Dacus.gypsoides	X
Ceratitis.lunata	X	Dacus.hapalus	X
Ceratitis.manjakatompo	X	Dacus.hargreavesi	X
Ceratitis.melanopus	X	Dacus.herensis	X
Ceratitis.nana	X	Dacus.hyalobasis	X
Ceratitis.neostictica	X	Dacus.iaspideus	X
Ceratitis.ovalis	X	Dacus.ikelenge	X
Ceratitis.paradumeti	X	Dacus.inclytus	X
Ceratitis.pinnatifemur	X	Dacus.jubatus	X
Ceratitis.sucini	X	Dacus.kakamega	X
Ceratitis.zairensis	X	Dacus.kariba	X
Dacus.abbabae	X	Dacus.katonae	X
Dacus.abditus	X	Dacus.linearis	X
Dacus.africanus	X	Dacus.lotus	X
Dacus.amberiens	X	Dacus.macer	X
Dacus.amphoratus	X	Dacus.madagascariensis	X
Dacus.apectus	X	Dacus.marshalli	X
Dacus.apiculatus	X	Dacus.maynei	X
Dacus.arabicus	X	Dacus.meladassus	X
Dacus.attenuatus	X	Dacus.merzi	X
Dacus.bakingiliensis	X	Dacus.mirificus	X
Dacus.basifasciatus	X	Dacus.nairobensis	X
Dacus.bequaerti	X	Dacus.namibiensis	X
Dacus.bidens	X	Dacus.nanus	X
Dacus.blepharogaster	X	Dacus.nigriscutatus	X
Dacus.bombastus	X	Dacus.nigrolateris	X
Dacus.brevis	X	Dacus.notalaxus	X
Dacus.carnesi	X	Dacus.opacatus	X
Dacus.carvalhoi	X	Dacus.pallidilatus	X
Dacus.chamun	X	Dacus.pamelae	X
Dacus.chapini	X	Dacus.panpyrrhus	X
Dacus.chiwira	X	Dacus.parvimaculatus	X
Dacus.clinophlebs	X	Dacus.pecropsis	X
Dacus.collarti	X	Dacus.persicus	X
Dacus.congoensis	X	Dacus.phantoma	X
Dacus.copelandi	X	Dacus.phimis	X
Dacus.croceus	X	Dacus.plagiatus	X

Dacus.pleuralis	X	Dacus.serratus	X
Dacus.pulchralis	X	Dacus.setilatens	X
Dacus.purpurifrons	X	Dacus.sphaerostigma	X
Dacus.pusillator	X	Dacus.spissus	X
Dacus.quilicii	X	Dacus.stentor	X
Dacus.radmirus	X	Dacus.stylifer	X
Dacus.rubicundus	X	Dacus.temnopterus	X
Dacus.rugatus	X	Dacus.trigonus	X
Dacus.ruslan	X	Dacus.umehi	X
Dacus.rutilus	X	Dacus.woodi	X
Dacus.sakeji	X	Dacus.xanthaspis	X
Dacus.scaber	X	Dacus.xanthopterus	X
Dacus.schoutedeni	X	Dacus.xanthopus	X
Dacus.segunii	X	Dacus.yemenensis	X
Dacus.seguyi	X		
Dacus.semisphaereus	X		

Chapter 2.2

Evolutionary trends in insect-host plant associations within a tropical fruit fly genus (Diptera: Tephritidae: *Ceratitis*).

Abstract

Using molecular data from three protein encoding genes and 49 species (98 specimens), we reconstructed the phylogeny of the genus *Ceratitis* (Diptera: Tephritidae) and investigated the evolution of host-plant specialization along the different recognized clades. Bayesian tree reconstructions supported previously proposed monophyletic lineages (i.e., *Pardalaspis*, *Pterandrus* subsection A and *Pterandrus* subsection B +*Ceratitis sensu strictu*) and two monophyletic subsections (A and B) of the subgenus *Ceratalaspis*. Reconstruction of ancestral character states for host plant relationships suggested that stenophagy (i.e., specialization on one plant genus) evolved repeatedly and independently within the genus *Ceratitis.* Six clades comprised more than one stenophagous species that share host genera and genus-specific main secondary metabolites, while at least in five different clusters (*Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris* feeders), a common polyphagous ancestor evolved into lineages with more restricted feeding preferences. We conclude that the observed phylogenetic patterns for stenophagous *Ceratitis* clusters are the result of an evolutionary process of ecological specialization to toxic hosts.

Introduction

Phytophagous insects are exposed to a wide variety of toxic secondary plant metabolites such as alkaloids, glucosinolates and furanucoumarins (Schoonhoven et al., 2006) that serve as primary defense mechanism against herbivory (Fraenkel, 1959). Concentrations of toxic secondary metabolites may vary considerably within and among plant species, and not all higher plants species contain such components (Evans, 2002). Some 'generalist' phytophagous species are only found on hosts that contain specific types of secondary metabolites and can therefore be considered 'specialists' with respect to host plant chemistry (Klausnitzer, 1983). For example, larvae of the cabbage white butterfly *Pieris rapae* are mainly restricted to cruciferous host plants (Brassicaceae) but may occasionally feed on *Tropaeolum* (nasturtium; Tropaeolaceae) or *Reseda* (Resedaceae) species too. Although these host plants belong to different families, they all contain glucosinolate secondary metabolites against which *Pieris rapae* larvae developed specific detoxification mechanisms (Renwick et al., 1999). Phytophagous species are generally restricted in the range of secondary metabolites they can tolerate (Jaenike, 1990), while different species that infest the same host plant may sometimes develop different detoxification or excretion mechanisms (Ratzka et al., 2002; Wittstock et al., 2004). As a consequence of the high diversity in secondary metabolites in host plants, and in metabolic pathways required to tolerate them, plant toxicity provides a significant barrier for host plant use by generalist species (Steppuhn et al., 2004) and may constitute a potential driver of their evolutionary ecology (Janz, 2005; Gotthard et al, 2005). Changes in host plant use, either as host range expansion or as shift to a new host with novel chemistry, can hence lead to phytophagous species radiation (Janz and Nylin, 1998). Such diversification through host shifts to related or unrelated but chemically similar host plants (Jaenike, 1990; Percy et al, 2004), is considered to be a potential source of ecological speciation in insects (Schluter, 2001; Funk et al, 2002).

Fruit flies (Diptera, Tephritidae) are considered an exemplary group of biological adaptation by exploiting phytophagy from an ancestral saprophagous origin (Drew & Yuval, 1999). This was only possible through key innovations such as the development of a plant tissue piercing ovipositor which enabled to exploit living tissue as a direct food source for the larval development. This resulted in an explosive resource exploitation by the larval stages of fruit flies whereby different strategies are recognized (Zwölfer, 1983) including broad-range and specialized exploiters of pulpy fruits. Here we test whether phylogenetic relationships among generalist (polyphagous) and specialist (stenophagous) fruit fly species of the genus *Ceratitis* are related to the secondary metabolite composition of their host plants. This genus comprises 95 described species placed in six different subgenera (*Ceratitis sensu stricto, Ceratalaspis* Hancock, *Pardalaspis* Bezzi, *Pterandrus* Bezzi, *Hoplolophomyia* Bezzi, and *Acropteromma* Bezzi) (De Meyer, 2005). While originally indigenous to the Afrotropic region, some species are adventive in other biogeographical regions where they may become agricultural pests (White and Elson-Harris, 1992). All *Ceratitis* species are considered true frugivores, i.e. they deposit their eggs in fruits and emerging larvae solely feed on these fruits. Host use within the genus ranges from highly polyphagous, with one species infesting over 20 host plant families, to highly stenophagous or monophagous, with one species infesting one single plant host genus or species, respectively (White *et al.*, 1999). Within Africa, a total of 48 families and more than 60 genera of host plants have been recorded (http://projects.bebif.be/enbi/fruitfly), a number of which comprise insecticidal secondary metabolites (Evans, 2002).

Morphological (De Meyer, 2000, 2005) and molecular (*period* (per), cytochrome oxidase I (COI), and NADH-dehydrogenase subunit 6 (ND6) genes; Barr and McPheron 2006; Barr and Wiegmann 2009) analyses of phylogenetic relationships earlier revealed that stenophagous species within the genus *Ceratitis* that infest the same genus of host plants tend to form distinct phylogenetic clades, while biochemical data (Coates Palgrave, 1983; Mabberley, 1997; Evans, 2002) confirmed that host plant species within these clades contain high concentrations of toxic metabolites. Here we

extend the phylogenetic tree of the genus *Ceratitis* with 52 specimens of 17 species and relate phylogenetic patterns with ecological data such as feeding strategy and main secondary metabolites of toxic hosts. Based on these results, we test if, and to what extent, evolutionary radiation of stenophagous (specialist) clades within the genus *Ceratitis* coincides with their ability to exploit toxic hosts that are not utilized by generalists within the same genus (De Meyer 2000, 2001). The objective of this study is to formally test relationships between host specificities and phylogenetic patterns of the genus *Ceratitis*.

Materials and Methods

Sampling and DNA sequencing

We analyzed a total of 98 *Ceratitis* specimens from 49 species belonging to five different subgenera (SM1, SM2) housed in the Royal Museum for Central Africa (Tervuren, Belgium), Two closely-related Afrotropical tephritids, *Capparimyia aenigma* (De Meyer and Freidberg) and *Capparimyia melanaspis* (Bezzi), were included as outgroups (De Meyer and Freidberg, 2005). Sequences were produced at the mitochondrial loci COI and ND6 and at the nuclear locus *period* (*per*). DNA was extracted from pinned and ethanol specimens using the DNeasy Blood and Tissue Kit (Qiagen) and following the manufacturer's protocol. DNA fragments were amplified using primers and protocols described in Barr and McPheron (2006), Barr et al. (2006) and Virgilio et al. (2008, 2009). Previously generated DNA sequences, as reported in Barr and McPheron (2006), were incorporated in the analysis.

Phylogenetic analysis

Nucleotide sequences were aligned using the default parameters of the IUB scoring matrix of ClustalW, as implemented in Bioedit 7.0 (Hall, 1999). Phylogenetic relationships were inferred through Bayesian tree reconstruction as implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). All analyses employed a cold chain

and three incrementally heated chains with T=0.1. Starting trees for each chain were random and tree searches were performed using default settings in MrBayes. Each metropolis coupled Markov Chain Monte Carlo (MCMC) was run for 3 million generations, with trees sampled every 1000 generations, and the first 1.5 million generations (1500 trees) were discarded as a burn-in. The chains converged to stable likelihood values <0.01 during the burning-in. Posterior probabilities (PP) were used to assess clade support. Analyses were run using the evolutionary models selected for each gene fragment by the Akaike information criterion of MrModeltest (Nylander, 2004). The general time reversible model (Tavaré, 1986) with invariant positions and gamma distributed rates (GTR+I+G) was used for COI and ND6, whereas the Hasegawa–Kishino–Yano model (Hasegawa et al., 1985) with gamma distributed rates (HKY+G) for the *period* gene fragment.

Congruence of phylogenetic signals provided by the mitochondrial (COI, ND6) and nuclear (*per*) partitions was evaluated through the Bayes factors method (Kass and Raftery, 1995, Nylander et al., 2004). The marginal likelihoods of different models of concatenation (COI+ND6+*per*, COI+ND6, COI+*per*, ND6+*per*, not concatenated) were estimated by linking the topologies of gene partitions in MrBayes 3.1. The harmonic means of the likelihood values of the MCMC samples were used to estimate the marginal likelihoods of each concatenation model. The marginal likelihoods of the fully linked topology of COI+ND6+*per* (B_0) and those of alternative models of concatenation (B_1) were compared by calculating $2\log_e(B_0/B_1)$ and evaluating the support provided to the alternative models according to Kass and Raftery (1995). All comparisons between the fully linked topology and alternative models of concatenation (including the fully unlinked topology) resulted in values of $2\log e(B_0/B_1)<2$ (Table 1), indicating that the phylogenetic signals provided by the three markers were not in conflict. Following this congruence, all three gene fragments were analysed as a single concatenated dataset of 1712bp.

Bayes factor analyses were initially implemented using specimens which produced sequences for all three markers only (*n*=61, 45 *Ceratitis* species). The topology of the

Bayesian tree generated by this method (SM3) was visually compared with a tree obtained when including specimens which produced only partial amplifications for one or two markers too (*n*=100, 49 *Ceratitis* species). As tree topologies obtained from both datasets were comparable, subsequent SH-tests and the reconstruction of the ancestral character states were based on the latter dataset. We performed a Maximum Parsimony (MP) analysis to test whether the molecular phylogeny obtained through Bayesian analysis was robust to different tree reconstruction methods, We therefore ran ten random addition replicates with tree-bisection-reconnection (TBR) branch swapping in PAUP* (Swofford, 2002). The number of rearrangements was limited to 10^8 per addition replicate. Characters were considered as "unord" with equal weights and gaps as missing data.

Evolution of host plant relationships

Ancestral character states of polyphagous and stenophagous species were reconstructed with Mesquite 2.01 (Maddison and Maddison, 2007). Stenophagous species infesting toxic hosts were assigned to one of six groups based on the main secondary metabolite composition of their host plants (Tab. 2). Maximum Likelihood (ML) reconstruction of ancestral states provided, for each node, a probability estimate for the evolution of host preferences. *A priori* hypotheses on the monophyly of stenophagous taxa on toxic hosts were tested with Shimodaira-Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999). The likelihood scores of Bayesian trees constrained for the monophyly of species feeding on host species *Cola*, *Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris* (Table 1) were compared to the likelihood score of the unconstrained Bayesian tree. The appropriate maximum likelihood model (and associated parameters) used for each SH test was specified in PAUP* (Swofford, 2002) following the Akaike information criterion of Modeltest 3.7 (Posada and Crandall, 1998). Constrains were tested for each host genus separately and for the combined set. Probability values of repeated comparisons were corrected for Type I errors using the False Discovery Rate procedure (Benjamini and Hochberg 1995).

Results

Phylogenetic relationships in the genus *Ceratitis*

Concatenation of data produced sequences of 1712bp with COI, ND6 and *per* contributing for 763, 482 and 467 bp, respectively. Bayesian analysis of the concatenated dataset (*n*= 100) divided the *Ceratitis* sequences into five well-supported (i.e. PP > 95%) monophyletic groups (Fig. 1). A total of 14 species (*n* = 22) were recovered within the *Ceratitis sensu strictu* + *Pterandrus section B* group (*C.s.s.*+*PT*(B)) which included two main multispecific clades (1 and 2) and a number of well-supported sub-clades (Fig. 1). *Pterandrus section A* (*PT* (A)) comprised 10 taxa (*n* = 18) and three main multispecific clades (3, 4, 5). Clade 5 included a subclade (5a) formed by *C. rubivora* and the FAR complex (Barr and McPheron 2006), with *C. rosa* + *C. rubivora* recovered as monophyletic (sub clade 5aa) and *C. anonae* and *C. fasciventris* being paraphyletic.

Ceratalaspis was not recognized as a monophyletic clade in the tree, but two clades that include *Ceratalaspis* species were recovered in the analyses. *Ceratalaspis* section A (*Cl* (A)) was defined by a well supported clade (Figure 1; (6)) including six species (*n*=26). This section included a well-supported subclade (6a) comprising *C. quinaria* and *C. silvestri*. Five additional *Ceratalaspis* species (n=13) formed a clade with *C.* (*Hoplolophomyia*) *cristata* (n=3). This clade [*Ceratalaspis* section B + *Hoplolophomyia* (*Cl* (B)+*H*)] comprised two well-supported clades (8 and 9). Six *Ceratalaspis* species (i.e., *Cl divaricata*, *Cl whartoni*, *Cl simi*, *Cl contramedia*, *Cl stictica* and *Cl lentigera*) remained unresolved in polytomies within the tree. The *Paradalaspis* group (*PD*) included 7 species (*n*=8), six of which grouped in clade 7. *Ceratalaspis* section B + *Hoplolophomyia* (*Cl* (B)+*H*) included 6 taxa (*n*=16) distributed within two well-supported clades (8 and 9).

MP analysis yielded 36,461 equally most-parsimonious trees (length=3,704). The topology resulting from the strict consensus MP tree (SM4) was similar to the

topology recovered with the Bayesian tree. Minor differences were observed for the *C.s.s.+PT*(B) group, for clades 1 and 2 and subclades 1a and 5aa.

Evolution of host plant relationships

Reconstruction of ancestral character states for host plant relationships showed that stenophagy is a homoplasious state within the genus *Ceratitis* (Fig. 2). Tolerance to toxic hosts evolved in common ancestors in five out of six groups with restricted host range, i.e. taxa infesting host plants belonging to the genera *Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris* (Fig. 3). Conversely, tolerance to toxic *Cola* host plants evolved separately in species *C. colae* and in *C. acicularis*/*C. penicillata*. On the other hand, *C. punctata* (as a sole polyphagous species that is recovered in the clade comprising the stenophagous *Tabernaemontana* feeders) appears as a character reversal.

The hypothesis of monophyly of the six groups of stenophagous species on toxic hosts was rejected when the constraints where tested as a combined set (Tab. 3). However, the unconstrained Bayesian tree did not differ significantly from each of the trees constrained for the monophyly of *Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris*, independently. The hypothesis of monophyly was rejected for species infesting *Cola* hosts. However, differences between constrained and unconstrained trees did not remain significant when probability levels were adjusted for multiple testing.

Figure 1. Phylogenetic tree of the fruit fly genus *Ceratitis* obtained from a concatenated Bayesian analysis of COI, ND6, and *per* genes (1712 bp, n=100). Groups (Css + Pt(B), …, Cl(B) + H), clades (1-9) and subclades (1a, …, 9b) with posterior probabilities > 95% are shown. For each *Ceratitis* specimen, the subgenus is indicated: Cl: *Ceratalaspis*, C: *Ceratitis s.s.*, H: *Hoplolophomyia*, PD: *Pardalaspis*, Pt: *Pterandrus*.

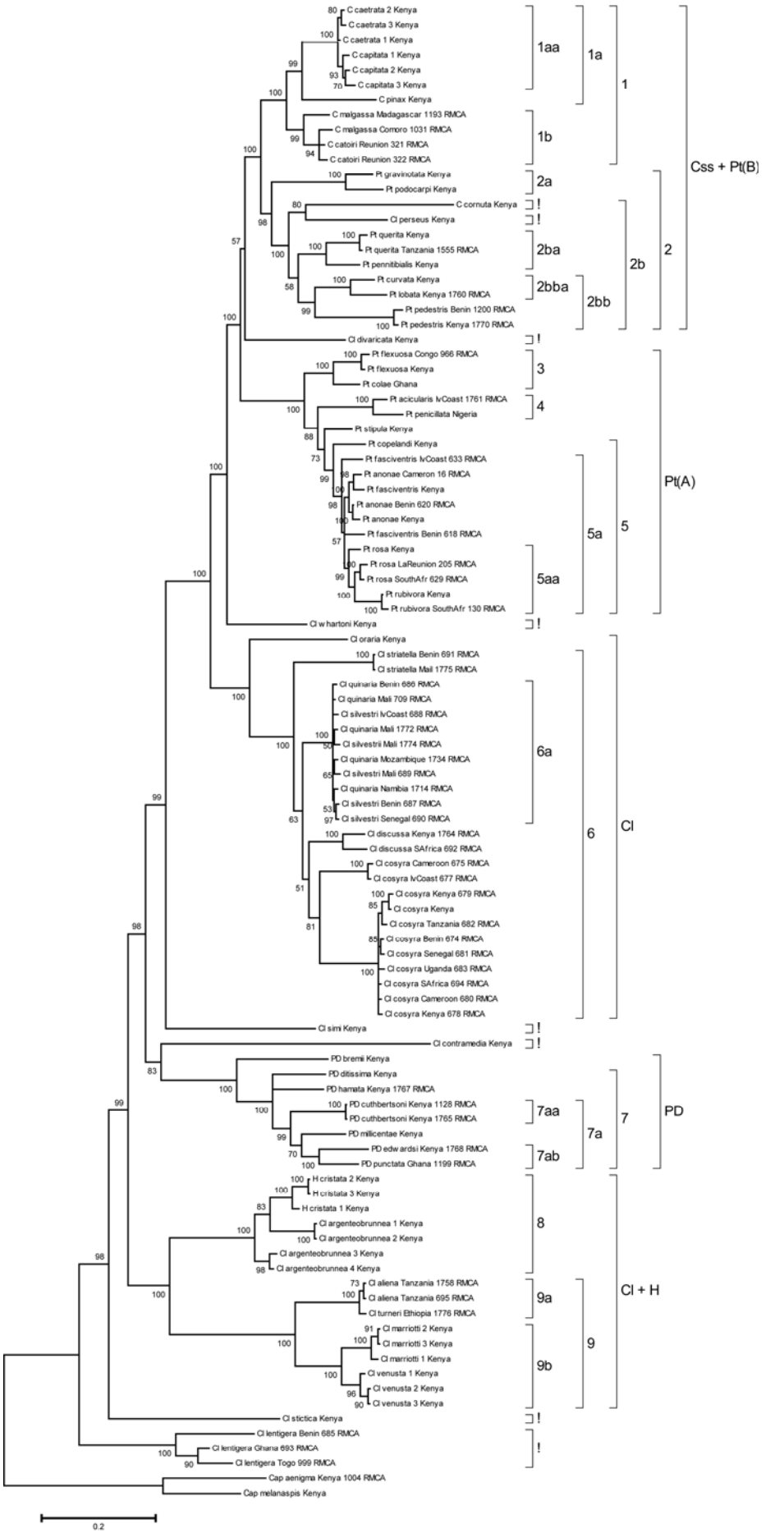
C caetrata 2 Kenya
C caetrata 3 Kenya
C caetrata 1 Kenya
C capitata 1 Kenya
C capitata 2 Kenya
C capitata 3 Kenya
C pinax Kenya
C malgassa Madagascar 1193 RMCA
C malgassa Comoro 1031 RMCA
C catoiri Reunion 321 RMCA
C catoiri Reunion 322 RMCA
Pt gravinotata Kenya
Pt podocarpi Kenya
C cornuta Kenya
Cl perseus Kenya
Pt querita Kenya
Pt querita Tanzania 1555 RMCA
Pt pennitibialis Kenya
Pt curvata Kenya
Pt lobata Kenya 1760 RMCA
Pt pedestris Benin 1200 RMCA
Pt pedestris Kenya 1770 RMCA
Cl divaricata Kenya
Pt flexuosa Congo 966 RMCA
Pt flexuosa Kenya
Pt colae Ghana
Pt acicularis IvCoast 1761 RMCA
Pt penicillata Nigeria
Pt stipula Kenya
Pt copelandi Kenya
Pt fasciventris IvCoast 633 RMCA
Pt anonae Cameron 16 RMCA
Pt fasciventris Kenya
Pt anonae Benin 620 RMCA
Pt anonae Kenya
Pt fasciventris Benin 618 RMCA
Pt rosa Kenya
Pt rosa LaReunion 205 RMCA
Pt rosa SouthAfr 629 RMCA
Pt rubivora Kenya
Pt rubivora SouthAfr 130 RMCA
Cl w hartoni Kenya
Cl oraria Kenya
Cl striatella Benin 691 RMCA
Cl striatella Mali 1775 RMCA
Cl quinaria Benin 686 RMCA
Cl quinaria Mali 709 RMCA
Cl silvestri IvCoast 688 RMCA
Cl quinaria Mali 1772 RMCA
Cl silvestrii Mali 1774 RMCA
Cl quinaria Mozambique 1734 RMCA
Cl silvestri Mali 689 RMCA
Cl quinaria Namibia 1714 RMCA
Cl silvestri Benin 687 RMCA
Cl silvestri Senegal 690 RMCA
Cl discussa Kenya 1764 RMCA
Cl discussa SAfrica 692 RMCA
Cl cosyra Cameroon 675 RMCA
Cl cosyra IvCoast 677 RMCA
Cl cosyra Kenya 679 RMCA
Cl cosyra Kenya
Cl cosyra Tanzania 682 RMCA
Cl cosyra Benin 674 RMCA
Cl cosyra Senegal 681 RMCA
Cl cosyra Uganda 683 RMCA
Cl cosyra SAfrica 694 RMCA
Cl cosyra Cameroon 680 RMCA
Cl cosyra Kenya 678 RMCA
Cl simi Kenya
Cl contramedia Kenya
PD bremi Kenya
PD ditissima Kenya
PD hamata Kenya 1767 RMCA
PD cuthbertsoni Kenya 1128 RMCA
PD cuthbertsoni Kenya 1765 RMCA
PD millicentae Kenya
PD edwardsi Kenya 1768 RMCA
PD punctata Ghana 1199 RMCA
H cristata 2 Kenya
H cristata 3 Kenya
H cristata 1 Kenya
Cl argenteobrunnea 1 Kenya
Cl argenteobrunnea 2 Kenya
Cl argenteobrunnea 3 Kenya
Cl argenteobrunnea 4 Kenya
Cl aliena Tanzania 1758 RMCA
Cl aliena Tanzania 695 RMCA
Cl turneri Ethiopia 1776 RMCA
Cl marriotti 2 Kenya
Cl marriotti 3 Kenya
Cl marriotti 1 Kenya
Cl venusta 1 Kenya
Cl venusta 2 Kenya
Cl venusta 3 Kenya
Cl stictica Kenya
Cl lentigera Benin 685 RMCA
Cl lentigera Ghana 693 RMCA
Cl lentigera Togo 999 RMCA
Cap aenigma Kenya 1004 RMCA
Cap melanaspis Kenya
0.2
1aa
1a
1
1b
2a
!
!
2ba
2b
2
2bba
2bb
Css + Pt(B)
!
3
4
5a
5
Pt(A)
5aa
!
6a
6
Cl
!
!
7aa
7a
7
PD
7ab
8
9a
9
Cl + H
9b
!
!

Table 1. Bayes factor analysis to verify inconsistencies among molecular markers. The harmonic means of marginal likelihoods of the fully concatenated dataset COI+ND6+*per* (B_0) and those of alternative models of concatenation (B_1) are compared by calculating $2\log_e(B_0/ B_1)$. Values <2 do not provide support to the alternative model (Kass and Raftery 1995).

		harmonic mean of marginal likelihoods	$2\log_e(B_0/ B_1)$
B_0:	COI+ND6+*per*	-18216.7	
alternative models (B_1):			
	not concatenated data	-17896.9	0.04
	COI+ND6	-17885.8	0.04
	COI+*per*	-17891.8	0.04
	ND6+*per*	-17777.3	0.05

Figure 2. Maximum likelihood reconstruction of the evolution of infestation strategies in the fruit fly genus *Ceratitis.* Taxa are coded as (i) polyphagous, (ii) stenophagous on toxic hosts, or (iii) stenophagous on non-toxic hosts (see Tab. 2). Ancestral character states are reconstructed based on the topology of the ingroup portion of the Bayesian tree (see Fig. 1). Pie diagrams on nodes indicate the relative likelihood of evolution of one of the three host use strategies. Triangles represent clades containing multiple specimens of the same species. For each *Ceratitis* specimen, the subgenus is indicated: Cl: *Ceratalaspis*, C: *Ceratitis s.s.*, H: *Hoplolophomyia*, PD: *Pardalaspis*, Pt: *Pterandrus*.

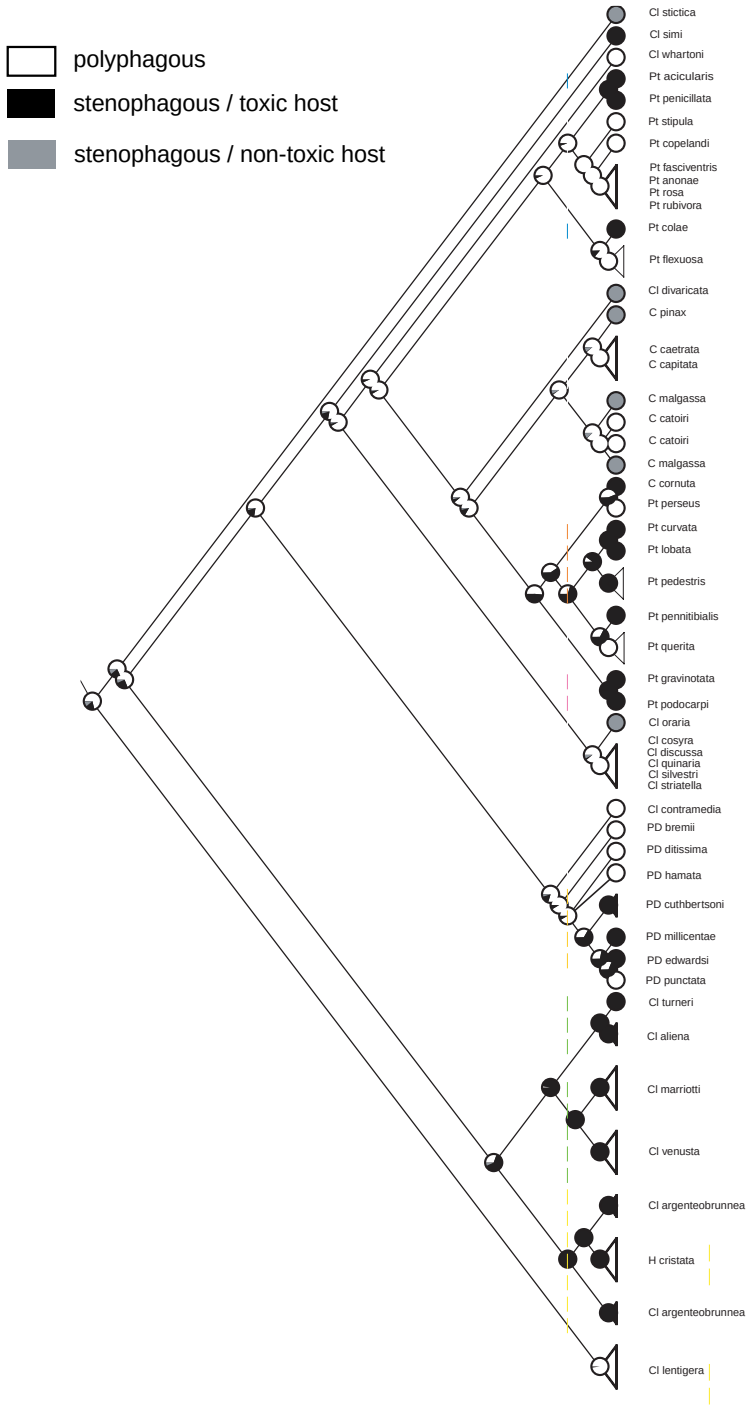
polyphagous
stenophagous / toxic host
stenophagous / non-toxic host
Cl stictica
Cl simi
Cl whartoni
Pt acicularis
Pt penicillata
Pt stipula
Pt copelandi
Pt fasciventris
Pt anonae
Pt rosa
Pt rubivora
Pt colae
Pt flexuosa
Cl divaricata
C pinax
C caetrata
C capitata
C malgassa
C catoiri
C catoiri
C malgassa
C cornuta
Pt perseus
Pt curvata
Pt lobata
Pt pedestris
Pt pennitibialis
Pt querita
Pt gravinotata
Pt podocarpi
Cl oraria
Cl cosyra
Cl discussa
Cl quinaria
Cl silvestri
Cl striatella
Cl contramedia
PD bremii
PD ditissima
PD hamata
PD cuthbertsoni
PD millicentae
PD edwardsi
PD punctata
Cl turneri
Cl aliena
Cl marriotti
Cl venusta
Cl argenteobrunnea
H cristata
Cl argenteobrunnea
Cl lentigera

Table 3. Shimodaira-Hasegawa tests comparing likelihood scores of unconstrained trees and trees constrained for the monophyly of species feeding on *Cola*, *Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana*, *Vespris*. Significant probability values after the False Discovery Rate correction (experimentwise α=0.05) are indicated with an asterisk.

constrain	-ln L	diff -ln L	p value
none	19665.22		
Cola	19693.74	28.52	0.02
Podocarpus	19660.64	4.58	0.23
Solanum	19656.55	8.67	0.09
Strychnos	19656.55	8.67	0.09
Tabernaemontana	19671.44	6.22	0.21
Vespris	19657.04	8.18	0.10
all	19706.77	41.55	0.01 *

Figure 3. Maximum likelihood reconstruction of the evolution of host use by stenophagous *Ceratitis* fruit flies in six genera of toxic host plants (*Cola*, *Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris*). Ancestral character states are reconstructed on the topology of the ingroup portion of the Bayesian tree (see Fig. 1). Pie diagrams on nodes indicate the relative likelihood of evolution of one of the host use strategies. Triangles represent clades containing multiple specimens of single species. See Fig. 1 for abbreviations of subgenera.

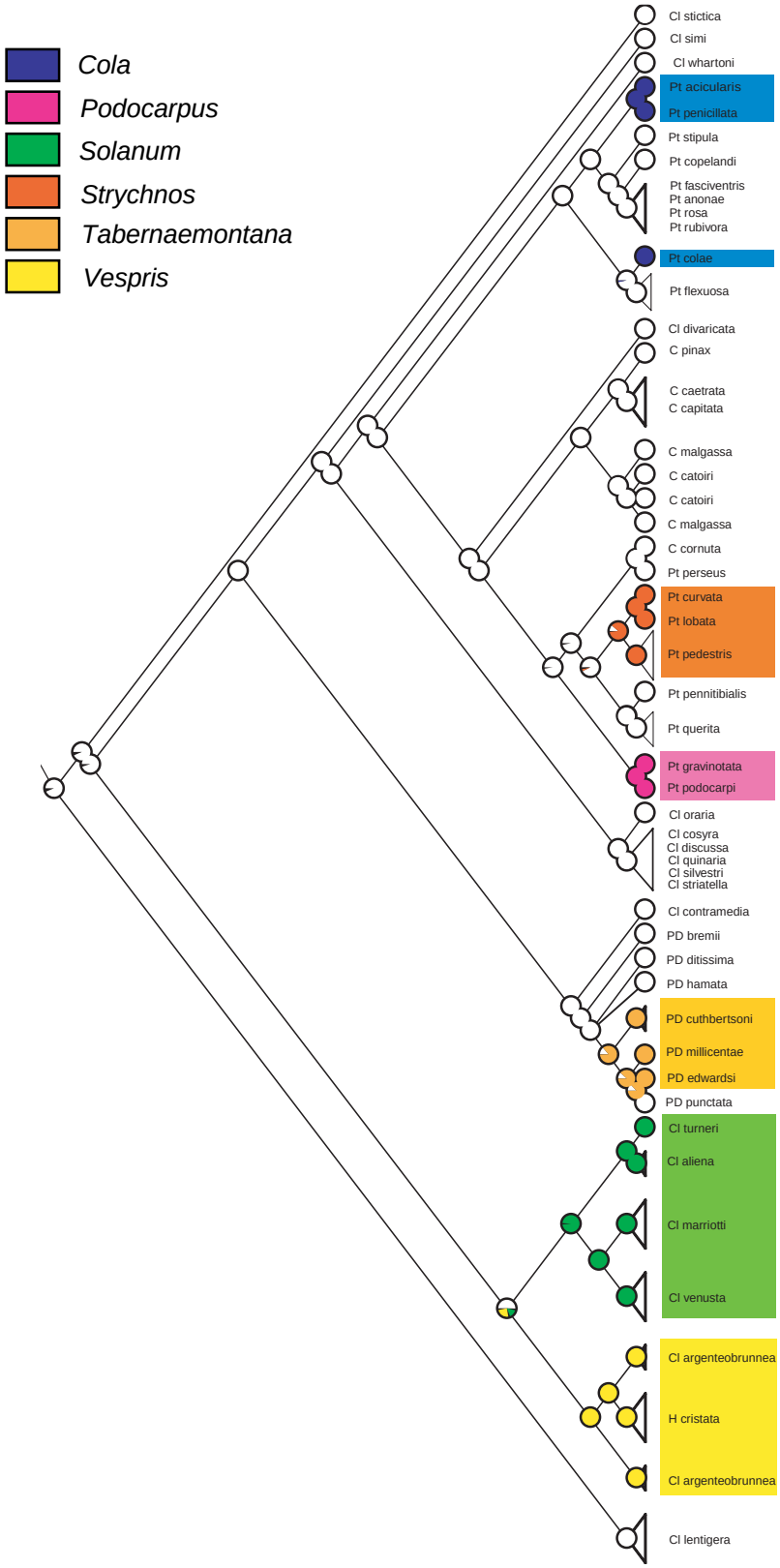
Cola
Podocarpus
Solanum
Strychnos
Tabernaemontana
Vespris
Cl stictica
Cl simi
Cl whartoni
Pt acicularis
Pt penicillata
Pt stipula
Pt copelandi
Pt fasciventris
Pt anonae
Pt rosa
Pt rubivora
Pt colae
Pt flexuosa
Cl divaricata
C pinax
C caetrata
C capitata
C malgassa
C catoiri
C catoiri
C malgassa
C cornuta
Pt perseus
Pt curvata
Pt lobata
Pt pedestris
Pt pennitibialis
Pt querita
Pt gravinotata
Pt podocarpi
Cl oraria
Cl cosyra
Cl discussa
Cl quinaria
Cl silvestri
Cl striatella
Cl contramedia
PD bremii
PD ditissima
PD hamata
PD cuthbertsoni
PD millicentae
PD edwardsi
PD punctata
Cl turneri
Cl aliena
Cl marriotti
Cl venusta
Cl argenteobrunnea
H cristata
Cl argenteobrunnea
Cl lentigera

Table 2. Host genera and main secondary metabolites (MSM) of stenophagous *Ceratitis* taxa feeding on toxic (T) and non toxic (NT) hosts. Toxic hosts involved in hypothesis testing are indicated in bold.

host genus	toxicity	MSM	reference	infesting flies
Cola	T	caffein	(Evans 2002)	*C. acicularis*
				C. colae
				C. penicillata
Podocarpus	T	norditerpene and bisnorditerpene dilactones	(Singh et al. 1979; Kubo et al. 1985)	*C. gravinotata*
				C. podocarpi
Solanum	T	solanine	(Nema et al. 2008)	*C. aliena*
				C. marriotti
				C. turneri
				C. venusta
Strychnos	T	strychnine	(Yin et al. 2007)	*C. curvata*
				C. lobata
				C. pedestris
Tabernaemontana	T	strictosidine glucosidase	(Luijendijk et al. 1996)	*C. cuthbertsoni*
				C. edwardsi
				C. lepida
				C. millicentae
Vepris	T	furoquinoline alkaloids	(Evans, 2002; Brader et al. 1996)	*C. argenteobrunnea*
				C. cristata
Acokanthera	T	ouabain	(Torrie et al. 2004; Abassy et al. 1977)	*C. simi*
Diospyros	T	ursolic and oleanolic acid	(Mallavadhani et al. 2003)	*C. pennitibialis*
Oxyanthus	T	cyanogenic glycosides	(Rockenbach et al. 1992)	*C. cornuta*
Craterispermum	NT	-		*C. stictica*
Ekebergia	NT	-		*C. divaricata*
Flagellaria	NT	-		*C. pinax*
Myristica	NT	-		*C. Malgassa*
Salacia	NT	-		*C.Oraria*

Discussion

The molecular phylogeny of the genus *Ceratitis* presented in this paper shows that the monophyletic subsection (Pt(A)) of the subgenus *Pterandus*, originally comprising species *Pt anonae, Pt copelandi, Pt fasciventris, Pt rosa, Pt rubivora*, *Pt stipula* (Barr and MacPheron 2006; Barr and Wiegmann 2009) also includes species *Pt acicularis*. Likewise, the paraphyletic subsection Pt(B), originally comprising species *Pt curvata*, *Pt gravinotata, Pt pennitibialis, Pt perseus, Pt podocarpi* and *Pt querita*, also comprises species *Pt lobata* and *Pt pedestris*. Two *Ceratitis s.s.* species that were not included in previous molecular phylogenies (*C. catoiri* and *C. malgassa*) are shown to form a monophyletic group with *C. caetrata*, *C. capitata* and *C. pinax*. Results of this study further support the monophyletic lineage of Pt(B) + C s.s. (see Barr and McPheron 2006), but not of Pt(A) and (Pt(B) + C s.s.), originally described by Barr and Wiegmann (2009) on the basis of six protein encoding gene fragments, due to an unresolved polytomy with species *Cl divaricata*. Lack of support for this latter monophyly may, however, be due to the lower number of markers analysed in the current study.

Results of this study further identify two main monophyletic lineages (Cl(A) and Cl(B)) and a number of paraphyletic outlier species (*Cl contramedia,Cl divaricata, Cl lentigera, Cl simi, Cl stictica* and *Cl whartoni*) within the subgenus *Ceratalaspis.* Lineage Cl(A) comprises species *Cl cosyra*, *Cl discussa*, *Cl oraria*, *Cl quinaria*, *Cl silvestri*, and *Cl striatella*, while lineage Cl(B) comprises species *Cl aliena*, *Cl argenteobrunnea*, *Cl marriotti* and *Cl turneri*, and forms a monophyletic lineage with *H cristata*. The subgenus *Ceratalaspis* was originally formulated as an entity comprised of species that could not be assigned to any of the other *Ceratitis* subgenera (De Meyer 2000) and, therefore by nature, presumed to be polyphyletic. Inclusion of species *PD cuthbertsoni*, *PD edwardsi*, *PD hamata,* and *PD punctata* further supports the monophyly of the subgenus *Pardalaspis* (Barr and McPheron 2006).

The proposed phylogeny shows a number of biological clades including stenophagous species which share host genera and genus-specific MSM. At least in five different

groups (*Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris* feeders), a common polyphagous ancestor gave raise to lineages with more specialized feeding strategy for toxic hosts. Whether host use specialization is a derived character state has been a matter of long debate (Holloway and Hebert, 1979; Moran, 1988; Janz and Nylin, 1998), in particular because a number of studies failed to detect directionality in the evolution of host preferences (e.g. Futuyama et al, 1995; Janz et al, 2001 butterflies) or provided evidence that generalist taxa evolved from specialized ancestors (e.g. Moran, 1988; Muller, 1996; Kelley and Farrell, 1998; Crespi and Sandoval, 2000; Scheffer and Wiegmann, 2000). In our study the directionality supports specialization as observed in the clades listed above. This trend is further supported by the phylogenetic patterns of the *Solanum* feeders which can be further subdivided into clades with narrower host specificities (*C. marriotti* / *C. venusta* feeding on *S. anguivi* and *C. aliena* / *C. turneri* infesting *S. nigrum*). The polyphagous species *C. punctata*, recovered among the *Tabernaemontana* feeders, appears as an exception to the general pattern. Yet, despite the polyphagous host range, the genus *Tabernaemontana* forms part of that range which implies the capability of detoxifying the *Tabernaemontana* MSM. This suggests that polyphagy in *C. punctata* is a character reversal.

Chemical cues determine which is the range of plants recognized as hosts and qualitative and quantitative differences in the composition of metabolites can lead to differential host ranges (Kühnle and Müller 2009). Chemical receptors of *Dacus* (Diptera: Tephritidae) respond to specific cues from appropriate hosts and determine its host range (Fitt 1986). In the same genus, host plant relationships and phylogenetic patterns are strictly related (Virgilio et al. 2009). Similarly, host choice of stenophagous *Ceratits* taxa seems to be related to genus specific MSM. Moreover, species specific MSM could further restrict the range of host specificities, as in the case of *C. marriotti* / *C. venusta* and *C. aliena* / *C. turneri*.

Acknowledgments

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Supplementary material

SM1: Species names, voucher numbers (RMCA: Royal Museum for Central Africa), sampling localities, subgeneric classification and GenBank accessions of 52 *Ceratitis* specimens (corresponding to 23 species) and a specimen of *Capparimyia aenigma* used for phylogenetic analyses. Specimens were sequenced at two mitochondrial (ND6, COI) and one nuclear (per) gene fragments. Asterisks indicate taxa not included in the previous phylogenetic analysis of Barr and McPheron (2006).

SM2: GenBank accessions of the mitochondrial (ND6, COI) and nuclear (per) sequences recovered from Barr and McPheron (2006) and used as part of the dataset for phylogenetic reconstructions. Species names, sampling localities and subgeneric classification are indicated for 46 Ceratitis specimens (corresponding to 33 species) and a specimen of *Capparimyia melanaspis.*

SM1

	species	voucher	country	subgenus	GenBank accessions ND6	COI	per
*	*C. aliena*	1758_RMCA	Tanzania	Ceratalaspis	GQ154336	GQ154425	GQ154385
*	*C. aliena*	695_RMCA	Tanzania	Ceratalaspis	GQ154337	GQ154426	-
	C. cosyra	674_RMCA	Benin	Ceratalaspis	GQ154341	-	GQ154388
	C. cosyra	675_RMCA	Cameroon	Ceratalaspis	GQ154342	GQ154429	GQ154389
	C. cosyra	680_RMCA	Cameroon	Ceratalaspis	GQ154343	-	GQ154390
	C. cosyra	677_RMCA	Ivory Coast	Ceratalaspis	GQ154344	-	-
	C. cosyra	678_RMCA	Kenya	Ceratalaspis	GQ154345	-	GQ154391
	C. cosyra	679_RMCA	Kenya	Ceratalaspis	GQ154346	-	GQ154392
	C. cosyra	694_RMCA	South Africa	Ceratalaspis	GQ154347	-	GQ154393
	C. cosyra	681_RMCA	Senegal	Ceratalaspis	GQ154348	GQ154430	GQ154394
	C. cosyra	682_RMCA	Tanzania	Ceratalaspis	GQ154349	GQ154431	GQ154395
	C. cosyra	683_RMCA	Uganda	Ceratalaspis	GQ154350	-	GQ154396
*	*C. discussa*	1764_RMCA	Kenya	Ceratalaspis	-	GQ154434	GQ154398
*	*C. discussa*	692_RMCA	South Africa	Ceratalaspis	GQ154353	GQ154435	GQ154399
*	*C. lentigera*	685_RMCA	Benin	Ceratalaspis	GQ154358	GQ154440	GQ154402
*	*C. lentigera*	693_RMCA	Ghana	Ceratalaspis	GQ154359	-	GQ154403
*	*C. lentigera*	999_RMCA	Togo	Ceratalaspis	GQ154360	-	GQ154404
*	*C. quinaria*	686_RMCA	Benin	Ceratalaspis	GQ154367	GQ154445	GQ154411
*	*C. quinaria*	1772_RMCA	Mali	Ceratalaspis	GQ154368	GQ154446	GQ154412
*	*C. quinaria*	709_RMCA	Mali	Ceratalaspis	GQ154369	-	GQ154413
*	*C. quinaria*	1734_RMCA	Mozambique	Ceratalaspis	GQ154370	GQ154447	GQ154414
*	*C. quinaria*	1714_RMCA	Namibia	Ceratalaspis	GQ154371	GQ154448	GQ154415
*	*C. silvestri*	687_RMCA	Benin	Ceratalaspis	GQ154376	GQ154452	GQ154416
*	*C. silvestri*	688_RMCA	Ivory Coast	Ceratalaspis	GQ154377	-	GQ154417
*	*C. silvestri*	689_RMCA	Mali	Ceratalaspis	GQ154378	-	GQ154419
*	*C. silvestri*	690_RMCA	Senegal	Ceratalaspis	GQ154379	-	GQ154420
*	*C. silvestri*	1774_RMCA	Mali	Ceratalaspis	GQ154375	GQ154451	GQ154418
*	*C. striatella*	691_RMCA	Benin	Ceratalaspis	GQ154380	GQ154453	GQ154421
*	*C. striatella*	1775_RMCA	Mali	Ceratalaspis	GQ154381	-	GQ154422
	C. turneri	1776_RMCA	Ethiopia	Ceratalaspis	GQ154382	-	GQ154423

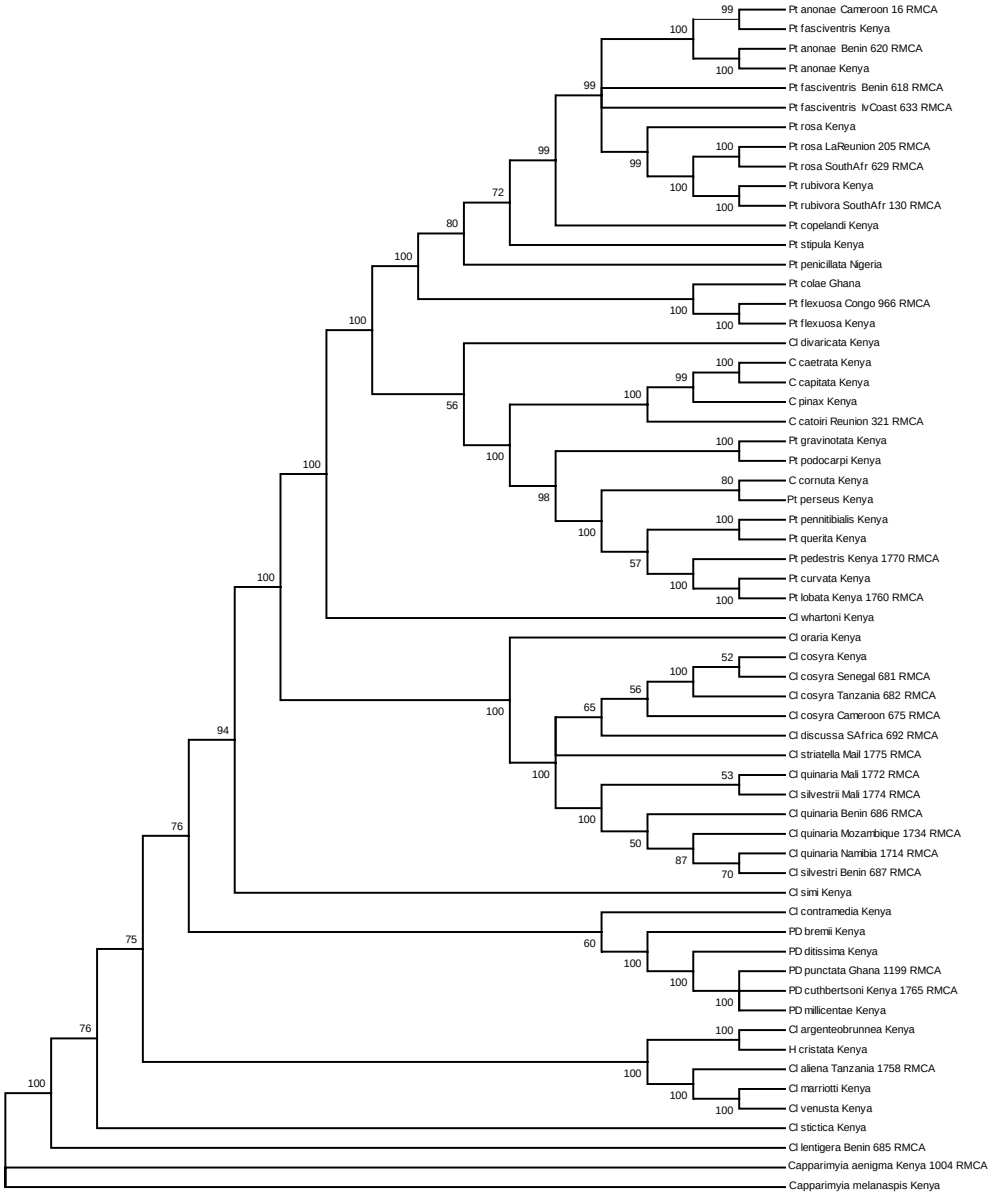
*	*C. catoiri*	321_RMCA	La Reunion	Ceratitis s.s.	GQ154340	GQ154428	GQ154386
*	*C. catoiri*	322_RMCA	La Reunion	Ceratitis s.s.	-	-	GQ154387
*	*C. malgassa*	1031_RMCA	Comoro	Ceratitis s.s.	GQ154362	-	-
*	*C. malgassa*	1193_RMCA	Madagascar	Ceratitis s.s.	-	GQ154406	GQ154442
*	*C. cuthbertsoni*	1128_RMCA	Kenya	Pardalaspis	GQ154351	GQ154432	GQ154397
*	*C. cuthbertsoni*	1765_RMCA	Kenya	Pardalaspis	GQ154352	GQ154433	-
*	*C. edwardsi*	1768_RMCA	Kenya	Pardalaspis	-	GQ154436	GQ154400
*	*C. hamata*	1767_RMCA	Kenya	Pardalaspis	GQ154357	-	-
*	*C. punctata*	1199_RMCA	Ghana	Pardalaspis	GQ154365	GQ154444	GQ154409
*	*C. acicularis*	1761_RMCA	Ivory Coast	Pterandrus	GQ154335	-	GQ154384
	C. anonae	16_RMCA	Cameroon	Pterandrus	GQ154338	EU276667	EU276838
	C. anonae	620_RMCA	Benin	Pterandrus	GQ154339	GQ154427	EU276853
	C. fasciventris	618_RMCA	Benin	Pterandrus	GQ154354	GQ154437	EU276830
	C. fasciventris	633_RMCA	Ivory Coast	Pterandrus	GQ154355	GQ154438	EU276833
	C. flexuosa	966_RMCA	Congo	Pterandrus	GQ154356	GQ154439	GQ154401
*	*C. lobata*	1760_RMCA	Kenya	Pterandrus	GQ154361	GQ154441	GQ154405
*	*C. pedestris*	1200_RMCA	Benin	Pterandrus	GQ154363	-	GQ154407
*	*C. pedestris*	1770_RMCA	Kenya	Pterandrus	GQ154364	GQ154443	GQ154408
	C. querita	1555_RMCA	Tanzania	Pterandrus	GQ154366	-	GQ154410
	C. rosa	205_RMCA	La Reunion	Pterandrus	GQ154372	EU276694	EU276865
	C. rosa	629_RMCA	SouthAfrica	Pterandrus	GQ154373	GQ154449	EU276869
	C. rubivora	130_RMCA	SouthAfrica	Pterandrus	GQ154374	GQ154450	EU276815
Capparimyia aenigma		1004_RMCA	Kenya		GQ154334	GQ154424	GQ154383

SM2

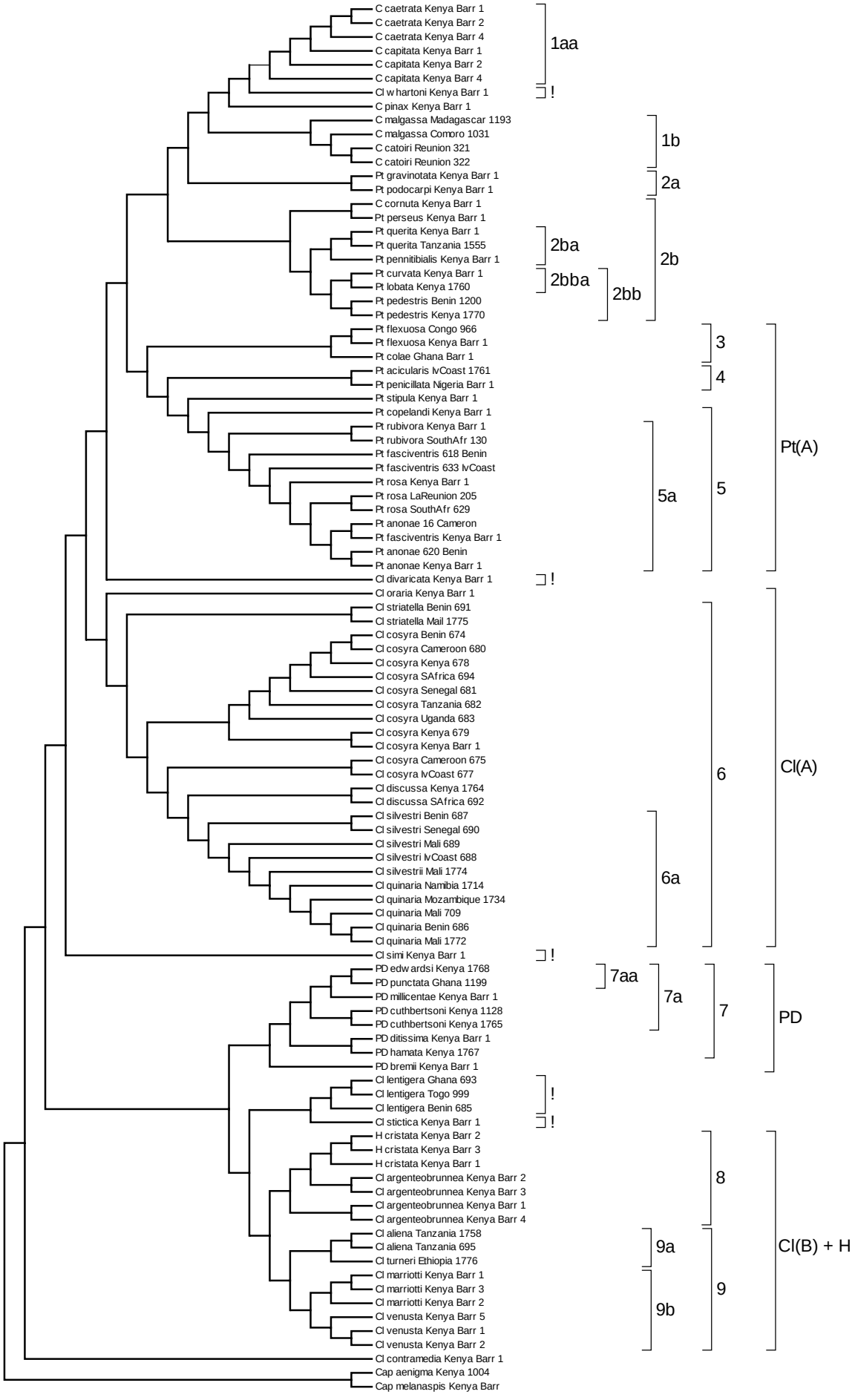
species	country	subgenus	GenBank accessions ND6	COI	per
C. argenteobrunnea	Kenya	Ceratalaspis	AY790587	AY788412	AY788363
C. argenteobrunnea	Kenya	Ceratalaspis	AY790588	-	-
C. argenteobrunnea	Kenya	Ceratalaspis	AY790589	-	-
C. argenteobrunnea	Kenya	Ceratalaspis	AY792149	-	-
C. contramedia	Kenya	Ceratalaspis	AY790595	AY788418	AY788369
C. cosyra	Kenya	Ceratalaspis	AY790553	AY788421	AY788372
C. divaricata	Kenya	Ceratalaspis	AY790555	AY788425	AY788376
C. marriotti	Kenya	Ceratalaspis	AY790556	AY788443	AY788394
C. marriotti	Kenya	Ceratalaspis	AY790557	-	-
C. marriotti	Kenya	Ceratalaspis	AY792135	-	-
C. oraria	Kenya	Ceratalaspis	AY790596	AY788416	AY788367
C. simi	Kenya	Ceratalaspis	AY790618	AY788439	AY788390
C. stictica	Kenya	Ceratalaspis	AY790559	AY788440	AY788391
C. venusta	Kenya	Ceratalaspis	AY792152	AY788442	AY788393
C. venusta	Kenya	Ceratalaspis	AY792152	-	-
C. venusta	Kenya	Ceratalaspis	AY792153	-	-
C. whartoni	Kenya	Ceratalaspis	AY790592	AY788429	AY788380
C. caetrata	Kenya	Ceratitis s.s.	AY790569	AY788414	AY788365
C. caetrata	Kenya	Ceratitis s.s.	AY792138	-	-
C. caetrata	Kenya	Ceratitis s.s.	AY790571	-	-
C. capitata	Kenya	Ceratitis s.s.	AY790546	AY788415	AY788366
C. capitata	Kenya	Ceratitis s.s.	AY792138	-	-
C. capitata	Kenya	Ceratitis s.s.	AY790549	-	-
C. cornuta	Kenya	Ceratitis s.s.	AY790614	AY788420	AY788371
C. pinax	Kenya	Ceratitis s.s.	AY790573	AY788433	AY788384
C. cristata	Kenya	Hoplolophomyia	AY790542	AY788422	AY788373

C. cristata	Kenya	Hoplolophomyia	AY792132	-	-
C. cristata	Kenya	Hoplolophomyia	AY792132	-	-
C. bremii	Kenya	Pardalaspis	AY790613	AY788413	AY788364
C. ditissima	Kenya	Pardalaspis	AY790558	AY788424	AY788375
C. millicentae	Kenya	Pardalaspis	AY790593	AY788430	AY788381
C. anonae	Kenya	Pterandrus	EU276725	AY788411	AY788362
C. colae	Ghana	Pterandrus	AY790607	AY788417	AY788368
C. copelandi	Kenya	Pterandrus	AY790606	AY788419	AY788370
C. curvata	Kenya	Pterandrus	AY790568	AY788423	AY788374
C. fasciventris	Kenya	Pterandrus	AY792158	AY788426	AY788377
C. flexuosa	Kenya	Pterandrus	AY790574	AY788427	AY788378
C. gravinotata	Kenya	Pterandrus	AY790615	AY788428	AY788379
C. penicillata	Nigeria	Pterandrus	AY790617	AY788432	AY788383
C. pennitibialis	Kenya	Pterandrus	AY790616	AY788431	AY788382
C. perseus	Kenya	Pterandrus	AY790594	AY788435	AY788386
C. podocarpi	Kenya	Pterandrus	AY790552	AY788434	AY788385
C. querita	Kenya	Pterandrus	AY790586	AY788436	AY788387
C. rosa	Kenya	Pterandrus	AY790575	AY788438	AY788388
C. rubivora	Kenya	Pterandrus	AY790585	AY788438	AY788389
C. stipula	Kenya	Pterandrus	AY790619	AY788441	AY788392
Capparimyia melanaspis	Kenya		AY790609	AY788456	AY788407

SM3: Tree obtained from the Bayesian analysis of 59 *Ceratitis* specimens (corresponding to 45 species) which produced sequences for all three markers used in the concatenated dataset COI + ND6 + *per* (1712 bp).



SM4: Majority rule consensus tree obtained from a Maximum Parsimony analysis of the concatenated dataset COI + ND6 + *per* (1712 bp, n=100). Groups, clades, subclades and subgeneric classification according to Fig. 1


C caetrata Kenya Barr 1
C caetrata Kenya Barr 2
C caetrata Kenya Barr 4
C capitata Kenya Barr 1
C capitata Kenya Barr 2
C capitata Kenya Barr 4
Cl w hartoni Kenya Barr 1
C pinax Kenya Barr 1
C malgassa Madagascar 1193
C malgassa Comoro 1031
C catoiri Reunion 321
C catoiri Reunion 322
Pt gravinotata Kenya Barr 1
Pt podocarpi Kenya Barr 1
C cornuta Kenya Barr 1
Pt perseus Kenya Barr 1
Pt querita Kenya Barr 1
Pt querita Tanzania 1555
Pt pennitibialis Kenya Barr 1
Pt curvata Kenya Barr 1
Pt lobata Kenya 1760
Pt pedestris Benin 1200
Pt pedestris Kenya 1770
Pt flexuosa Congo 966
Pt flexuosa Kenya Barr 1
Pt colae Ghana Barr 1
Pt acicularis IvCoast 1761
Pt penicillata Nigeria Barr 1
Pt stipula Kenya Barr 1
Pt copelandi Kenya Barr 1
Pt rubivora Kenya Barr 1
Pt rubivora SouthAfr 130
Pt fasciventris 618 Benin
Pt fasciventris 633 IvCoast
Pt rosa Kenya Barr 1
Pt rosa LaReunion 205
Pt rosa SouthAfr 629
Pt anonae 16 Cameron
Pt fasciventris Kenya Barr 1
Pt anonae 620 Benin
Pt anonae Kenya Barr 1
Cl divaricata Kenya Barr 1
Cl oraria Kenya Barr 1
Cl striatella Benin 691
Cl striatella Mail 1775
Cl cosyra Benin 674
Cl cosyra Cameroon 680
Cl cosyra Kenya 678
Cl cosyra SAfrica 694
Cl cosyra Senegal 681
Cl cosyra Tanzania 682
Cl cosyra Uganda 683
Cl cosyra Kenya 679
Cl cosyra Kenya Barr 1
Cl cosyra Cameroon 675
Cl cosyra IvCoast 677
Cl discussa Kenya 1764
Cl discussa SAfrica 692
Cl silvestri Benin 687
Cl silvestri Senegal 690
Cl silvestri Mali 689
Cl silvestri IvCoast 688
Cl silvestrii Mali 1774
Cl quinaria Namibia 1714
Cl quinaria Mozambique 1734
Cl quinaria Mali 709
Cl quinaria Benin 686
Cl quinaria Mali 1772
Cl simi Kenya Barr 1
PD edw ardsi Kenya 1768
PD punctata Ghana 1199
PD millicentae Kenya Barr 1
PD cuthbertsoni Kenya 1128
PD cuthbertsoni Kenya 1765
PD ditissima Kenya Barr 1
PD hamata Kenya 1767
PD bremii Kenya Barr 1
Cl lentigera Ghana 693
Cl lentigera Togo 999
Cl lentigera Benin 685
Cl stictica Kenya Barr 1
H cristata Kenya Barr 2
H cristata Kenya Barr 3
H cristata Kenya Barr 1
Cl argenteobrunnea Kenya Barr 2
Cl argenteobrunnea Kenya Barr 3
Cl argenteobrunnea Kenya Barr 1
Cl argenteobrunnea Kenya Barr 4
Cl aliena Tanzania 1758
Cl aliena Tanzania 695
Cl turneri Ethiopia 1776
Cl marriotti Kenya Barr 1
Cl marriotti Kenya Barr 3
Cl marriotti Kenya Barr 2
Cl venusta Kenya Barr 5
Cl venusta Kenya Barr 1
Cl venusta Kenya Barr 2
Cl contramedia Kenya Barr 1
Cap aenigma Kenya 1004
Cap melanaspis Kenya Barr
1aa
!
1b
2a
2ba
2bba
2bb
2b
3
4
5a
5
Pt(A)
!
6a
6
Cl(A)
!
7aa
7a
7
PD
!
!
8
9a
9b
9
Cl(B) + H

Section III

Stress and development in *Ceratitis* fruit flies

Chapter 3.1

Hybridisation between two polyphagous fruit fly species (Diptera: Tephritidae) causes sex-biased reduction in developmental stability

Abstract

When hybridisation modifies the genetic constitution of individuals or populations, the stability of phenotypic development may either decrease or increase, pending on the divergence in the gene systems controlling development between the hybridizing taxa, i.e. on the relative effects of outbreeding depression and heterosis. In genetically closely-related species, strong heterotic effects are less likely to occur, hence hybridization may be expected to cause an overall decrease in developmental stability (DS) due to the disruption of co-adapted gene complexes. To test this hypothesis, we experimentally crossed two closely-related species of *Ceratitis* fruit flies and compared multiple-trait fluctuating asymmetry (FA, a measure of DS) in male and female offspring between parental species and two crossbred types. All traits measured play an important role in the fanning and buzzing behaviour associated with male courtship in *Ceratitis* or are located on body parts that do so. As predicted, hybrid offspring developed more asymmetrically than offspring of either parental species - most notably in meristic traits - and the increase in FA was consistently and significantly stronger in females than in males. The fact that males buffered their development more efficiently than females is in concordance with the presumed between-sex variation in functionality, hence cost of asymmetry, of the measured traits. Absence of a similar sex difference in DS among parental offspring is believed to result from overall weak association between DS and FA in absence of genetic stress, due to the random nature of the underlying processes that trigger asymmetric development.

Introduction

An organism's genome is generally considered to comprise a set of harmoniously collaborating genes, combined and preserved by selective processes over the evolutionary history of populations. The resulting genetic balance (often referred to as genomic co-adaptation) reflects non-additive effects both acting within loci (dominance and overdominance) and between loci (epistasis) (Alibert & Auffray, 2003). While individual-level effects of these genomic processes are generally studied in terms of fertility and survival (Clarke & McKenzie 1987, 1992; Barton & Hewitt 1989; Arnold & Hodges 1995; Barton & Shpak 2000), the precision by which genes and gene complexes mutually interact may also affect the level of developmental stability (DS) of an individual, defined as the ability to buffer its developmental pathways against random perturbations of genetic or environmental origin (e.g. Graham 1992, Clarke 1993; Auffray, Dabat & Alibert, 1999). In synergy with other homeostatic processes, DS constitutes a major process involved in the reduction of phenotypic variation between and within environments (Debat & David, 2001; Davidowitz & Nijhout, 2003). The degree of DS of an organism (or population) is most commonly estimated by its level of fluctuating asymmetry (FA), defined as the magnitude of small random deviations from perfect symmetry in one or more bilateral traits (Palmer, 1994). Within an organism, both body sides develop according to the same genetic program and (most often) under identical environmental conditions. Hence, any difference in development (size, shape, number of ornaments) between both sides of a bilateral trait can be assumed to reflect the intrinsic variability of the developmental process (Zakharov, 1992).

When molecular processes such as introgression, mutation or intense directional selection modify the genetic constitution of individuals or populations, their levels of DS may either decrease or increase, depending on the relative importance of two interrelated properties, i.e. genomic balance and genomic heterozygosity (Clarke, Oldroyd & Hunt, 1992; Clarke 1993). Disruption of co-adapted gene complexes due to the merging of different genomes (e.g. in the case of hybridization) generally leads to a

reduction of DS. In contrast, increased genome-wide heterozygosity in hybrid offspring can be expected to enhance DS, either through allelic dominance (masking the expression of deleterious recessive alleles) or increased efficiency of particular biochemical or physiological processes (overdominance; Vrijenhoek & Lerman, 1982; Leary & Allendorf & Knudsen, 1984; Mitton & Grant, 1984; Mitton, 1993). Although relationships between DS and heterozygosity were long considered to be theoretically and practically flawed (*e.g.* Clarke, 1993), the number of studies supporting increased DS in inter(sub)specific hybrids is currently growing (Alibert & Auffray, 2003).

At present, however, there is no theoretical framework that predicts under which conditions merging of parental genomes in hybrids will lead to developmental outbreeding or heterosis, apart from the assumption that the outcome depends on the degree of divergence in the gene systems controlling development in the hybridizing taxa (Vrijenhoek & Lerman, 1982). Under high genomic similarity of parental taxa, increase in genome-wide heterozygosity in their hybrid offspring can be expected to be low, making strong heterotic effects unlikely. Under increasing genetic divergence, heterozygosity in hybrids can be expected to increase, but so may the level of incompatibility of the gene systems controlling development. Reported relationships between the taxonomic status of hybridizing groups and DS of hybrids are highly heterogeneous, suggesting that the links between divergence at protein loci (genetic distance) and divergence at regulatory loci (i.e. assessed by DS) are not consistently straightforward. The strength of relationships between genetic stress and DS may further differ between traits exhibiting different levels of functional importance. As functionally important traits are likely to be under stronger canalizing selection than less important ones, one should expect to find the former to exhibit a lower degree of FA because higher asymmetry would have stronger fitness consequences (Palmer & Strobeck, 1986; Clarke, 1998; Debat *et al.*, 2000, Garnier *et al.*, 2006). Such trait-specific variation in selection pressures has been invoked as one mechanism reducing between-trait correlations in asymmetry at the individual level (Clarke 1998).

In this study we compared levels of developmental stability, by means of multiple-trait FA, between parentals and lab-reared F1 hybrids of two closely-related polyphagous fruit fly species (*Ceratitis (Pterandrus) rosa* (Karsch) and *Ceratitis (Pterandrus) fasciventris* (Bezzi)). Both species belong to a monophyletic clade within the subgenus *Pterandrus* (Barr & McPheron, 2005) and, together with *Ceratitis (Pterandrus) anonae* (Graham), form the FAR species complex (Barr *et al.*, 2006). Although males of both species can be differentiated by secondary sexual traits, females are very difficult to identify at species level (De Meyer & Freidberg, 2006). Recent molecular work also revealed little genetic divergence between the three taxa, rendering them difficult or impossible to separate by using nuclear and mitochondrial markers such as COI, 16S, ND6 and Period (Barr *et al.*, 2006; Virgilio M, unpublished data). Apart from their phenotypic and genotypic resemblance, FAR species further share a highly similar host profile, showing distinct fruit host partitioning in comparison to related polyphagous species such as *Ceratitis capitata* (Wiedemann) and *Ceratitis cosyra* (Walker) (Copeland *et al.*, 2006). By selecting two parental species that show low genetic divergence, we expected weak heterosis in interspecific hybrids that cannot balance possible outbreeding effects due to the disruption of co-adapted gene complexes. Within this framework of increased genetic stress, we compared levels of FA in parental and hybrid individuals in a suite of metric and meristic traits that are associated with male courtship behaviour in *Ceratitis*. As no such functionality is known for females, we predicted a weaker increase in FA in male than in female hybrid offspring compared to parental offspring, due to stronger developmental buffering in the former.

Material and Methods

Study species

Both study species belong to the Afrotropical fruit fly genus *Ceratitis* (Diptera: Tephritidae). Most representatives of this genus (sized 4-8 mm) have pictured wings (yellow to brown bands) and several are of economic importance (White & Elson-Harris, 1992). Males bear different sexually dimorphic characters such as modified orbital bristles or setal ornamentation of the legs (De Meyer, 1999). Species *C. rosa* and *C. fasciventris* have a largely allopatric distribution and only co-occur in the Central Highlands of Kenya, possibly following a recent introduction of *C. rosa* from the coastal region (Copeland *et al.*, 2006). Interspecific hybrids have not (yet) been observed in the wild.

For this study, parental individuals originating from continuous breeding lines regularly supplemented with wild congeners from allopatric populations (*C. rosa* from South Africa and *C. fasciventris* from Kenya) and hybrid F1 offspring were provided by the International Atomic Energy Agency (Seibersdorf, Austria). IAEA has a long-standing expertise in breeding fruit fly species under (near) optimal conditions for Sterile Insect Techniques. Females of both species are nearly impossible to separate, although scutellar markings can be used to some degree. However, parental stocks of both species originated from allopatric areas, hence comprising single species lineages that were reared in isolation. As individuals of opposite sex were selected from each population during crossbreeding, the species-specific status of each individual was unequivocally known.

Adults were kept in cages of 11cm x 11cm x 15cm and fed a standard artificial diet of sugar and yeast (G. Franz, pers. com.). Females were allowed to lay eggs into grapes which were then transferred to Petri dishes with standard artificial larval diet until full larval development. Third instars were collected in sand where they pupariated. We measured male and female offspring of two crossbred types, i.e. male *C. fasciventris* x

female *C. rosa* (hereafter referred to as H1) and male *C. rosa* x female *C. fasciventris* (H2), and compared these with male and female offspring of parental crosses of *C. fasciventris* (P1) and *C. rosa* (P2).

Measurements

A total of 394 specimens from the following eight groups were measured: P1 males (51 individuals), P1 females (52 individuals), P2 males (50 individuals), P2 females (34 individuals), H1 males (51 individuals), H1 females (50 individuals), H2 males (53 individuals) and H2 females (53 individuals). In each individual, seven bilateral traits were measured, four of which were metric and three were meristic. Metric traits comprised femur length of the second and third leg pairs (Figs. 1C-D) and two distance measures between wing veins (Fig. 1A). Meristic traits comprised the number of setae on the caudal tibia side of the second leg pair (Fig. 1E) and on the rostral tibia side of the third leg pair (Fig. 1F), and the number of setulae on the ventral side of the R1 wing vein (Fig. 1B). All measured traits play an important role in the fanning and buzzing behaviour associated with male courtship in *Ceratitis* species or are situated on body parts that do so (Eberhard 1999; Yuval & Hendrichs 1999; Quilici *et al.*, 2002).

All measurements and counts were carried out by the first author. Prior to measurement, specimens were preserved in 98% ethanol. Alcohol immersion can result in setae becoming detached from the main structures, although less so than in dry preserved specimens, but only after rough handling. However, the setal implant remains discernible and can be used for meristic counts. Furthermore, sclerotized structures and wing surfaces are not known to alter in size due to alcohol conservation. Right and left wings and second and thirds leg pairs were dissected, briefly immersed in glycerol and subsequently mounted on microscope slides in glycerol gelatin. Images were captured by a Leica MZ12s stereomicroscope (Leica Microsystems ltd., CH-9435 Heerbrugg, Switzerland) equipped with a Toshiba 3CCD camera and *Auto-Montage pro* software (Syncroscopy version 5.01, Synoptics ltd., Beacon House, Cambridge,

UK). Optical magnification amounted 10 x 6.3 for all measurements and counts on legs, and 10 x 5.0 for all measurements and counts on wings (additional optical magnification 2x). Linear measurements were performed with *Image Pro Plus* software (Media Cybernetics, Silver Spring, MD, USA) using the *Best fit line* commando based of the number of pixels per line (one pixel per unit). Measurements and counts of either side of the bilateral traits were made repeatedly and independently (sequence left-right-left-right or right-left-right-left). Seven outliers (on a total of 2758 measurements) were visually detected and excluded from further analysis.

FA estimation

We carried out mixed regression analysis with Restricted Maximum Likelihood (REML) parameter estimation to separate real asymmetry (signed FA; i.e. left minus right trait value corrected for trait size) from measurement error (ME) (Van Dongen, Molenbergs & Matthysen, 1999a). The significance of FA was obtained from likelihood ratio tests while directional asymmetry (DA) was tested by *F*-tests with adjusted denominator degrees of freedom (Satterthwaite's procedure; Verbeke & Molenberghs, 2003). FA cannot be separated with high statistical power from antisymmetry (AS). However, a leptokurtic distribution of the signed FA values is generally considered indicative for the presence of antisymmetrical individuals in a population (Palmer & Strobeck 1992; Van Dongen 1998; see Rowe, Repasky & Palmer, 1997 for a comparable approach). Following D'Agostino & Stephens (1986), we compared the kurtosis of the signed FA values in each subsample against a critical *k*-value calculated as:

$$k = \left(\sum \left(X_i - \overline{X}\right)^4 / \left(N \times SD^4\right)\right) - 3$$

with N = sample size, $\overline{X}$ = sample mean, X_i = value for individual i, SD = standard deviation of the sample.

Unbiased individual FA estimates were calculated as deviations of random slopes from the fixed effects slope in the mixed regression model described above (Van Dongen *et al.*, 1999a), and standardized to zero mean and unit variance. To examine whether signed FA values of different traits were correlated at the individual level (i.e. indicative for correlated development; Van Dongen, Sprengers & Lofstedt, 1999b), we calculated Pearson's correlation coefficients.

Hypothesis testing

As all analyses involved repeated measurements at individual level (i.e. seven traits measured per fly), we performed mixed regression analysis with repeated-measure structure using Proc Mixed in SAS (Littell *et al.*, 1996). While such routine generally produces results comparable to those obtained with composite indices of FA (see Leung, Forbes & Houle, 2000), pseudo-replication is avoided because the degrees of freedom reflect the number of individuals rather than the number of traits by individuals (Van Dongen *et al.*, 1999a). Based on Akaike's Information Criteria, a compound symmetric model best described the covariance among the seven traits. The estimated covariance ($\rho\sigma^2 = 0.013$) indicated weak within-individual correlation in unsigned FA values. The initial model included standardized FA as dependent variable and factors 'sex', 'hybridisation' (parental or hybrid), 'type' (meristic or metric) and the two-factor interactions 'sex*hybridisation' and 'type*hybridisation' as fixed effects. To test whether additive and interactive effects were consistent among traits and/or groups, we added factors 'trait' (nested within 'type'), 'group' and the 'trait*sex', 'trait*hybridisation', 'trait*sex*hybridisation', 'group*sex' and 'group*type' interactions as random effects to the model. Applying a stepwise backward selection procedure, the statistical significance of variance components was tested by likelihood ratio tests. Fixed effects were tested by *F*-tests, adjusting the denominator degrees of freedom by Satterthwaite's formula (Verbeke & Molenberghs, 2003).

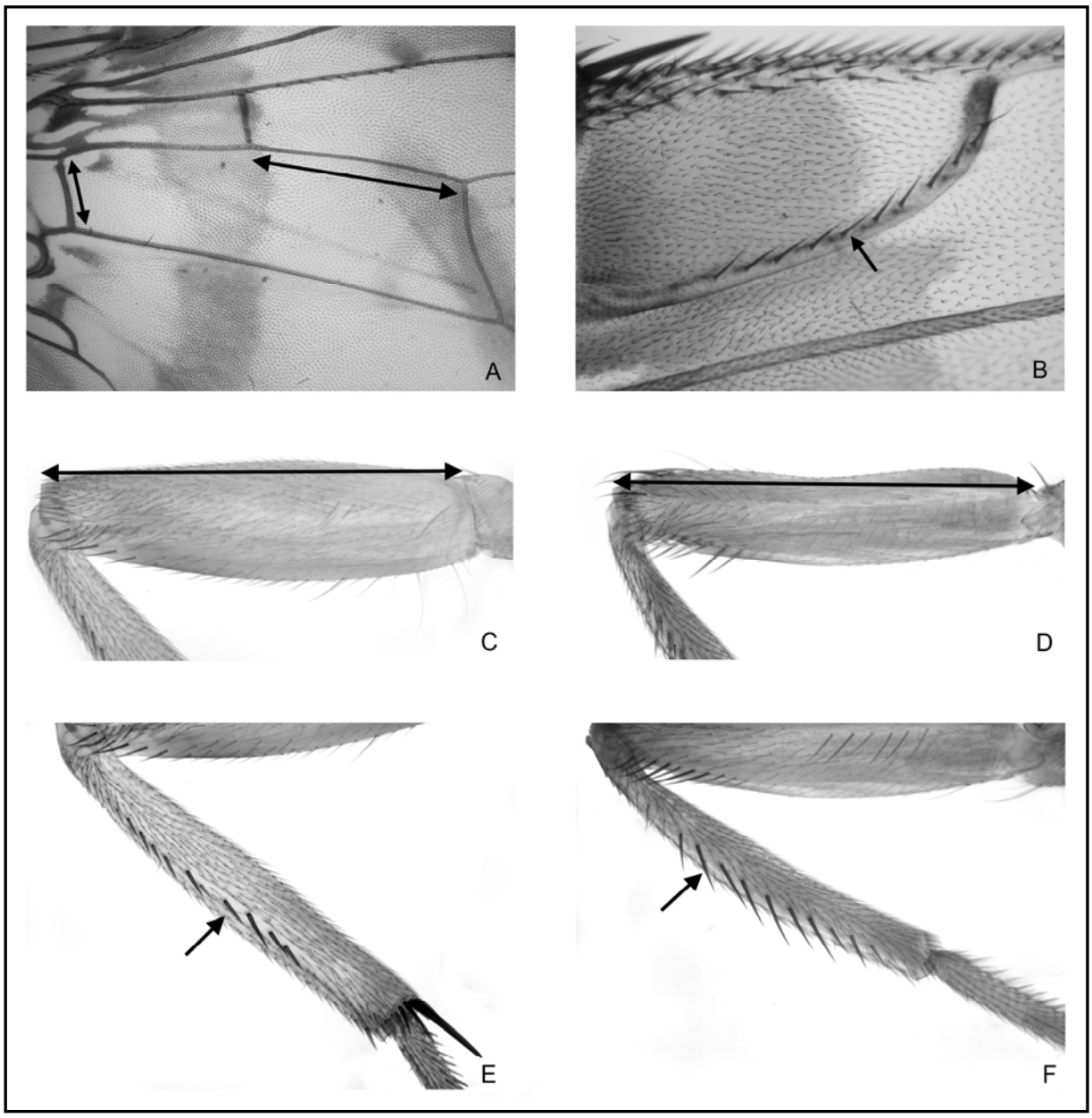


Figure 1. Metric and meristic traits measured on 394 offspring of parental and hybrid crosses of two related *Ceratitis* species. (A) distance measures between veins; (B) setulae on ventral side of R1 wing vein; (C) femur length on second leg; (D) femur length on third leg; (E) number of setae on caudal side of second tibia; (F) number of setae on rostral side of third tibia.

Results

Variance components and distribution of the signed FA

After Bonferroni correction for multiple testing, mean FA levels significantly differed from zero in two out of 56 group-by-trait combinations only, i.e. femur length of the third leg pair in female P2 offspring and the distance measure between wing veins (vl2) in male P1 offspring (Table 1, all other P>0.05). As both traits did not show directional asymmetry in any of the other groups, and as the number of P2 females was small relative to the other groups, both subsets were retained for further hypothesis testing after correction of the corresponding FA values for directional asymmetry. The platykurtic distribution of the signed FA values did not support the presence of antisymmetric individuals in any of the populations (Table 1, all values > critical *k*). Based on the comparison of REML values of models with and without random side effect, FA estimates were highly significant for all group-by-trait combinations after Bonferroni correction (Table 1, all $V_{FA}>V_{ME}$ and all P<0.001). As signed FA values were not significantly correlated between traits (Pearson's correlation: all P>0.05 after Bonferroni correction), traits were assumed to have developed independently from each other.

Effects of hybridization on unsigned FA

On average, hybrid offspring were significantly more asymmetric than parental offspring (Table 2). The level of increase in FA was significantly higher for females than for males (Fig. 2) as supported by a significant sex*hybridization interaction (P=0.035). This pattern was largely consistent among traits (trait*sex*hybridization interaction not significant, Table 2). While male and female offspring did not differ in FA averaged across traits, the significant random trait*sex interaction (P=0.012) indicated variation across traits in difference between sexes. After Bonferroni correction, trait-by-trait comparison showed significant sex effects in three out of seven traits in parental offspring (number of setae on the caudal tibia side of the

second leg pair and both distance measures between wing veins) and one trait in hybrid offspring (number of setae on the caudal tibia side of the third leg pair). The direction of the difference between sexes was, however, not consistent among traits (Fig. 2). The level of increase in FA in hybrid offspring was significantly higher for meristic than for metric traits (Fig. 2), as supported by a significant type*hybridization interaction (P=0.0001, Table 2).

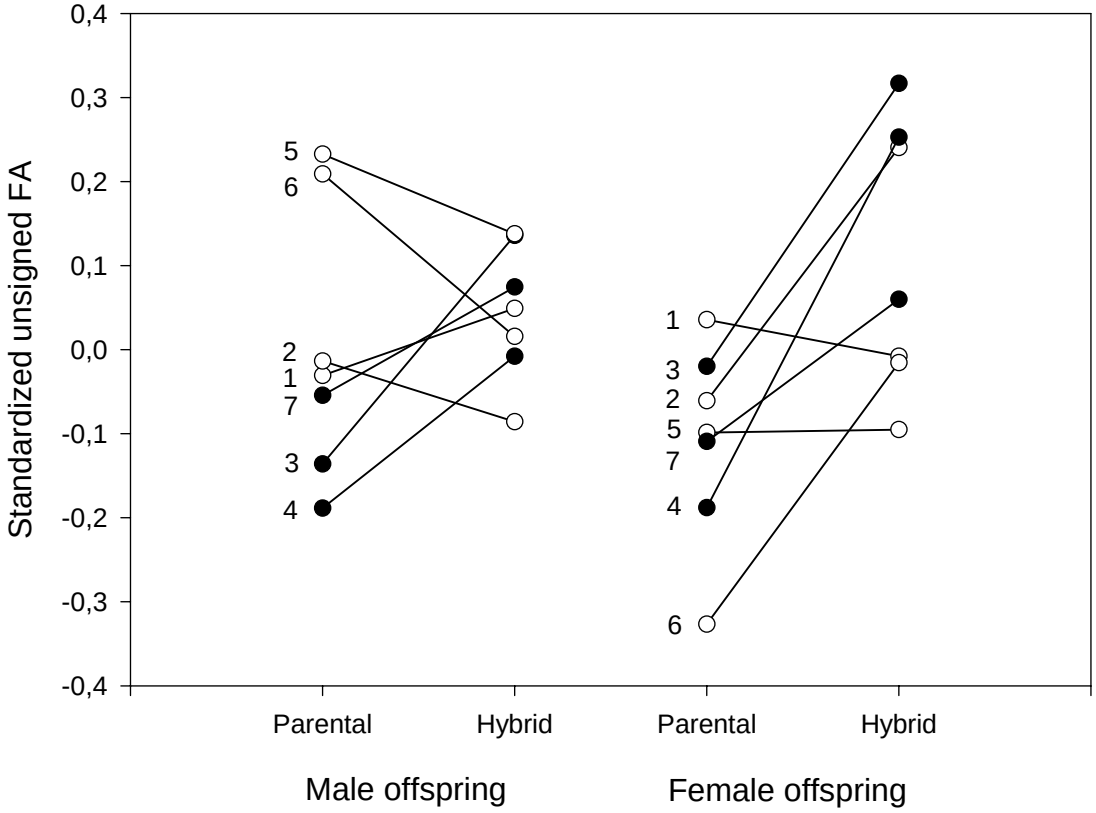


Figure 2. Mean standardized unsigned FA values in male and female offspring of parental and hybrid crosses of two related *Ceratitis* species. Open circles refer to metric traits, filled circles refer to meristic traits; (1) femur length of the second leg pair; (2) femur length of the third leg pair; (3) number of setae on the caudal tibia side of the second leg pair; (4) number of setae on the rostral tibia side of the third leg pair; (5) wing vein measure 1; (6) wing vein measure 2; (7) number of setulae on the ventral side of the R1 wing vein (see text for details on traits).

Table 1. Variance components and distribution of the signed FA in male and female offspring of parental and hybrid crosses of two related *Ceratitis* species.

n=number of individuals, V_{ind} = variance in individual trait size, V_{FA} = variance in signed FA, V_{ME} = variance in measurement error; (*) values computed as the difference between the mean values of both sides.

Type	Trait	n	df	F	P(F)	V_{ind}	V_{FA}	V_{ME}	χ^2	$P(\chi^2)$	*kurtosis*
P1 ♂	fem2	51	50	0.02	0.90	1989.13	393.41	172.78	25.6	<0.0001	1.76
P1 ♂	fem3	51	50	0.37	0.55	170.31	63.82	11.62	63.3	<0.0001	1.96
P1 ♂	vl1	51	50	0.37	0.55	170.31	63.82	11.62	63.3	<0.0001	1.96
P1 ♂	vl2	51	50	9.16	**0.004**	1630.20	267.50	12.01	163.9	<0.0001	-0.11
P1 ♂	tib2s	51					0.00 (*)				1.92
P1 ♂	tib3s	51	50	0.03	0.86	1.48	1.42	0.005	410.5	<0.0001	0.98
P1 ♂	vlst	51					1.33 (*)				-0.16
P1 ♀	fem2	52	51	3.44	0.07	1762.76	291.59	103.33	33.4	<0.0001	19.29
P1 ♀	fem3	50	46.1	4.04	0.0502	1912.58	407.79	85.79	51.8	<0.0001	1.26
P1 ♀	vl1	52	49	0.61	0.44	144.82	50.57	26.17	19.2	<0.0001	3.93
P1 ♀	vl2	51	49	8.40	**0.01**	2216.05	187.67	12.6464	129.5	<0.0001	9.06
P1 ♀	tib2s	52	51	1.17	0.28	1.34	1.95	0.03	272.2	<0.0001	0.43
P1 ♀	tib3s	52	51	0	0.96	1.47	1.87	0.04	228.9	<0.0001	-0.59
P1 ♀	vlst	52					1.20 (*)				4.16
P2 ♂	fem2	50	49	3.86	0.06	1811.20	412.27	39.65	102.1	<0.0001	0.22
P2 ♂	fem3	49	45.1	3.67	0.06	1957.05	490.31	72.12	68.8	<0.0001	0.83
P2 ♂	vl1	48	46	1.06	0.31	302.68	417.88	33.86	107.1	<0.0001	1.21
P2 ♂	vl2	48	45	1.53	0.22	1240.07	347.62	10.66	181.9	<0.0001	30.02
P2 ♂	tib2s	50	46.5	0.02	0.90	1.16	0.28	0.06	54.1	<0.0001	3.58
P2 ♂	tib3s	50	49	0.58	0.45	1.06	2.11	0.08	175.8	<0.0001	1.43
P2 ♂	vlst	50					0.89 (*)				-0.6
P2 ♀	fem2	34	33	0.07	0.79	3053.34	3277.17	52.01	173.1	<0.0001	0.47
P2 ♀	fem3	34	31.6	15.22	**0.001**	2284.44	1036.68	72.78	80.5	<0.0001	-0.32
P2 ♀	vl1	32	30.2	1.2	0.28	111.58	36.29	10.67	24.4	<0.0001	-0.94
P2 ♀	vl2	32	29.6	3.78	0.06	1658.09	295.51	23.77	71.1	<0.0001	-0.17
P2 ♀	tib2s	34	32.5	0.29	0.59	0.68	0.34	0.07	35	<0.0001	4.81
P2 ♀	tib3s	34	33	1.55	0.22	0.98	1.45	0.09	92.5	<0.0001	0.31
P2 ♀	vlst	34					0.85 (*)				-0.08
H1 ♂	fem2	51	49.5	2.68	0.11	1746.50	1814.58	185.37	98.7	<0.0001	1.35
H1 ♂	fem3	50	49	0.59	0.45	1128.04	319.56	66.72	54.8	<0.0001	-0.42
H1 ♂	vl1	50	49	0.16	0.69	93.16	42.24	20.86	21.8	<0.0001	0.12
H1 ♂	vl2	51	50	0	0.97	1012.16	194.39	55.87	41	<0.0001	44.16
H1 ♂	tib2s	51	50	0.85	0.36	1.45	1.60	0.27	66.8	<0.0001	0.46
H1 ♂	tib3s	51	50	1.01	0.32	1.34	2.66	0.15	147.2	<0.0001	0.4
H1 ♂	vlst	51					1.21 (*)				-0.13
H1 ♀	fem2	50	47.2	0.62	0.44	1308.61	208.36	84.64	26.7	<0.0001	0.2
H1 ♀	fem3	50	46.2	5.82	**0.02**	884.14	377.9	59.694	13.6	<0.0001	-0.13
H1 ♀	vl1	50	49	3.93	0.053	85.26	24.97	8.59	33.2	<0.0001	-0.25
H1 ♀	vl2	50	49	1.82	0.18	1136.66	201.88	11.21	144	<0.0001	-0.3
H1 ♀	tib2s	50	49	2.65	0.11	0.75	2.04	0.14	127.1	<0.0001	1.54
H1 ♀	tib3s	50	49	2.62	0.11	1.13	2.70	0.05	240.5	<0.0001	-0.46
H1 ♀	vlst	50					1.19 (*)				-0.39
H2 ♂	fem2	52	51	0.25	0.62	2374.61	381.02	30.12	121.1	<0.0001	2.88
H2 ♂	fem3	51	48.5	0.01	0.94	2047.68	934.15	239.52	45.1	<0.0001	1.92
H2 ♂	vl1	52	51.1	0.57	0.46	342.66	32.20	7.67	50.1	<0.0001	0.14
H2 ♂	vl2	52	51	0.06	0.80	1526.41	209.76	11.95	147.6	<0.0001	0.74
H2 ♂	tib2s	52	51	2.63	0.11	0.76	1.32	0.12	112	<0.0001	1.38
H2 ♂	tib3s	52	51	1.4	0.24	1.37	3.18	0.13	176.3	<0.0001	-0.61
H2 ♂	vlst	53					1.28 (*)				-0.64
H2 ♀	fem2	53	52	0.59	0.45	2094.97	668.34	76.85	95.4	<0.0001	0.74
H2 ♀	fem3	52	47.6	0.84	0.36	2631.45	739.86	79.62	95.7	<0.0001	2.86
H2 ♀	vl1	52	51	2.1	0.15	251.36	36.69	9.48	46.6	<0.0001	-0.31
H2 ♀	vl2	52	50.2	1.9	0.17	1299.65	219.60	15.34	129.4	<0.0001	5.98
H2 ♀	tib2s	53	52	0.14	0.71	0.82	2.01	0.10	158.7	<0.0001	0.62
H2 ♀	tib3s	52	51	0.43	0.52	1.30	3.20	0.06	244.9	<0.0001	0.01
H2 ♀	vlst	52					1.27 (*)				-0.26

Table 2. Test of fixed and random effects on individual estimates of unsigned FA in male and female offspring of parental and hybrid crosses of two related *Ceratitis* species.

Source	Statistic	Significance
Fixed effects		
Sex	$F_{1,11.7} = 0.03$	$P = 0.86$
Type	$F_{1,11} = 0.02$	$P = 0.89$
Hybridisation	$F_{1,403} = 12.75$	$\mathbf{P = 0.0004}$
Sex*Hybridisation	$F_{1,435} = 4.50$	$\mathbf{P = 0.035}$
Type*Hybridisation	$F_{1,2322} = 32.37$	$\mathbf{P = 0.0001}$
Random effects		
Trait (Type)	$\sigma^2 = 0.0000$	$P = 1.00$
Group	$\sigma^2 = 0.0002$	$P = 0.98$
Trait (Type)*Sex	$\sigma^2 = 0.0089$	$\mathbf{P = 0.012}$
Trait (Type)*Hybridisation	$\sigma^2 = 0.0059$	$P = 0.16$
Group*Sex	$\sigma^2 = 0.0000$	$P = 1.00$
Group*Type	$\sigma^2 = 0.0001$	$P = 0.99$
Trait (Type)*Sex*Hybridisation	$\sigma^2 = 0.0000$	$P = 1.00$

Discussion

When *C. rosa* and *C. fasciventris* were experimentally crossed under laboratory conditions, hybrid offspring showed a higher degree of asymmetrical development than offspring of each of the parental species, in particular in meristic traits. Such pattern supports the hypothesis that heterotic effects cannot balance outbreeding effects due to the disruption of co-adapted gene complexes in interspecific offspring bred from closely-related species. Since both species are morphologically and genetically very closely related and share the same natural host profile (Copeland *et al.*, 2006), their hybrids can be expected to share similar environmental (host) requirements under artificial breeding. Nevertheless, the average decrease in DS among hybrids may have partly reflected increased environmental stress as well. Traditionally, natural hybridization has been considered maladaptive, leading to natural selection against hybrid offspring (Mayr, 1963). However, Arnold & Emms (1998) provide an overview of examples where hybrids, despite demonstrating a lower fitness, gave rise to new evolutionary lineages. In Tephritid fruit flies, that often show a close relationship and specificity with host plant species, this may eventually lead to host plant shifts. While such mechanism has recently put forward to explain homoploid hybrid speciation in the genus *Rhagoletis* (Schwarz *et al.*, 2005), the evolutionary consequences of hybridization in the genus *Ceratitis* (comprising both polyphagous and strictly stenophagous clades; De Meyer, 2001, 2005) remain unknown.

The inverse effect of hybridization on developmental stability was stronger in female than in male offspring, indicating that individuals of the latter sex more efficiently buffered their development against destabilizing effects. This pattern was consistent among traits. Similarly, female hybrids of *Drosophila melanogaster* (Meigen) x *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae) were more asymmetric than male hybrids (Markow & Ricker, 1991). Because natural selection can be expected to favor mechanisms that maximize fitness, the development of traits that contribute more directly to an individual's reproductive success or survival should be buffered

more effectively against random perturbations (Palmer & Strobeck, 1986; Clarke, 1998). Likewise, if the functional significance of a given set of traits varies between males and females, associations between developmental stability and stress can be expected to differ between sexes (see Hunt *et al.*, 1998 for a similar argument when asymmetry in secondary sexual traits reduces male mating success). Since all traits analyzed in this paper play an important role in the fanning and buzzing associated with male courtship behaviour in *Ceratitis* species (while no such behaviour has been observed in females; Eberhard, 1999; Yuval & Hendrichs, 1999; Quilici *et al.*, 2002), fitness costs due to asymmetric development, if any, can be expected to be higher for males than for females. Studies on other organisms detected opposite sex-differences, e.g. due to higher levels of energetic or nutritional stress in the larger (male) sex (Clutton-Brock *et al*., 1985; Sheldon *et al*., 1998) or presumed antagonistic effects of male steroid hormones on developmental buffering (Zuk, 1990; Folstad & Karter, 1992; Sheldon *et al*., 1998).

While the observed difference in DS under genetic stress was, indeed, in the direction predicted by the hypothesis of trait functionality, a similar pattern might have been expected among parental offspring. The fact that such pattern did not emerge may reflect a problem common to many FA studies, i.e. the overall weak association between DS and FA due to the random nature of the underlying processes that trigger asymmetric development (Lens *et al.*, 2002). Because of this weak association, developmentally unstable individuals may display low FA under favourable conditions (environmentally, genetically, or both) when the presumed levels of developmental noise are low. Under enhanced levels of genetic or environmental stress, individuals face increasing energetic problems to maintaining high levels of DS (Palmer, 1994; Leung & Forbes, 1997) and developmentally unstable ones become unmasked by elevated levels of FA. Similarly, populations of birds and butterflies exposed to increasing levels of environmental stress showed stronger relationships between genetic stress and FA (Lens *et al*., 2000; Kark *et al*. 2001). In our study, hybrid offspring were raised in a common environment with the parental species, thereby

largely ruling out additional interactive effects between environmental and genetic stress.

Absence of significant correlations in signed FA among different leg and wing measures suggests that traits represented developmentally independent units, and hence, were not morphologically integrated (Van Dongen *et al.*, 1999b). Morphological integration has formerly been observed in the mandibles of the mouse jaw (Leamy, 1993), limbs of nine mammalian species (Hallgrimsson, 1998), legs of two moth species (Van Dongen, Sprengers & Lofstedt, 1999b) and traits related to locomotion in greenfinches *Carduelis chloris* (Karvonen *et al*, 2003). Although mechanistic explanations are generally lacking, correlations in signed FA values are assumed to confound the interpretation of patterns in the unsigned FA (Van Dongen, Sprengers & Lofstedt, 1999b). The estimated covariance between the unsigned FA values was low too, indicating that single-trait FA only poorly predicted organism-wide asymmetry due to the absence of an Individual Asymmetry Parameter (IAP; Palmer & Strobeck, 1986; Clarke, 1998; Debat *et al.*, 2000). Whereas the described relationships between FA, hybridization and sex were largely consistent among traits, effect sizes were on average larger for meristic than for metric traits. While this is consistent with the outcome of a meta-analysis examining effects of trait type, habitat, stressor and taxonomic group on the strength of FA-stress relationships (Lens *et al.*, 2001), it remains unknown how general this pattern is, due to the small number of experimental studies that directly compare relationships between both trait types. It has been suggested that shape FA may comprise a more sensitive measure of stress than metric or meristic traits (Auffray *et al*, 1996, Knierim *et al*., in press), however, empirical confirmation is still required. In the absence of a conceptual framework that predict which (types of) traits are most likely prone to developmental perturbations from external (environmental) or internal (genetic) origin, results from this study therefore endorse the use of FA estimates based on multiple body traits, at least some of which should be meristic.

Apart from trait type and non-additive effects of different types of stressors, heterogeneity in reported relationships with FA is believed to result from heterogeneity in statistical accuracy (Lens *et al.*, 2002). We here applied a statistical routine that combines information from a suite of developmentally uncorrelated traits without perpetrating pseudo-replication, and that allowed us to distinguish FA from measurement error and other types of bilateral asymmetry that are known to hamper proper interpretation of patterns in developmental stability (Lens *et al.*, 2002). Hopefully, increased use of analogous protocols will further reduce the degree of statistical noise and, hence, increase the likelihood of revealing true underlying patterns between population and individual levels of DS, genetic and environmental stress, and fitness.

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Solanum anguivi, Nairobi, Kenya © Nathalie Erbout

Solanum mauritianum, Nairobi, Kenya © Nathalie Erbout

Chapter 3.2

Variation in glycoalkaloid concentrations between and within two Solanum hosts of *Ceratitis* fruit flies (Diptera, Tephritidae)

Abstract

Many plant species pursue defense strategies against phytophagous organisms through the production of secondary compounds that act as a repellent or impair the attacker's development. The glycoalkaloids, α-chaconine and α-solanine are such compounds, and occur in variable concentrations in plant species of the genus *Solanum,* representatives which act as hosts for polyphagous or stenophagous fruit flies of the genus *Ceratitis*. We tested a presumed relationship between glycolalkaloid presence and stenophagy by defining the α-chaconine and α-solanine concentration in a known stenophagous (*Solanum anguivi*) and in a polyphagous (*Solanum mauritianum*) host through HPLC analyses. The highest overall concentration was observed in the stenophagous host in comparison with the polyphagous one, although the concentration gradually decreases during ripening process of the former.

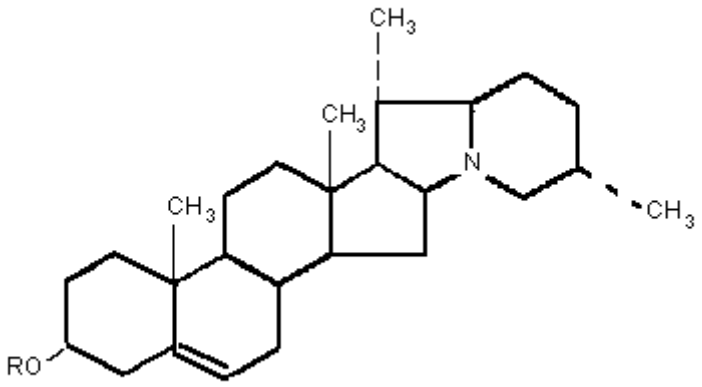
Introduction

During their evolutionary history, many plant species developed defense mechanisms against phytophagous and pathogen selection pressures, such as the production of secondary compounds that impair herbivore development or repel phytophagous attacks. Glycoalkaloids of *Solanum* species are discerned as such toxic secondary metabolites (Cipollinin & Levey, 1997). They sometimes occur as paired glycoalkaloids, i.e. two compounds based on a common aglycone but differing in their carbohydrate moiety (as α-chaconine and α-solanine). As such, they may act synergistically, subsequently orchestrating the level of toxicity, whereby the alkaloid concentration deters with progressing ripening of the *Solanum* fruit (Roddick, 1989). Many plants of the genus *Solanum* act as host plants for Afrotropical fruit flies (Diptera: Tephritidae) of the genus *Ceratitis* (De Meyer et al., 2002). Within this fruit fly genus, patterns of stenophagy are closely associated with the toxicity of their respective host plants, and the evolutionary radiation of stenophagous clades has been hypothesized to be driven by the ability of stenophagous species to exploit toxic hosts (De Meyer, 2001; De Meyer, 2005).

By seeking evidence for the presumed relationship between plant toxicity and stenophagy in the genus *Ceratitis*, we extracted glycoalkaloids from ripe *Solanum mauritianum* fruits and four consecutive ripening stages of *S. anguivi* fruits, two well known host species of *Ceratitis* fruit flies (De Meyer et al., 2002). While *S. mauritianum* acts as a host for different polyphagous species, *S. anguivi* is infested only by the stenophagous species *C. marriotti* Munro and *C. venusta* (Munro) (De Meyer et al., 2002). If toxicity levels play a role in stenophagy, we expect to find higher glycoalkaloid concentrations in *Solanum anguivi* than in *S. mauritianum*. Since it is generally assumed that toxic secondary metabolites are lost, bound, or otherwise deactivated during the process of ripening (cf. Eltayeb and Roddick, 1985) and successful infestation time (in ripe fruits) by *Ceratitis* species will be in accordance with this process, it is of interest to determine a possible variation of concentration during ripening of *S. anguivi* fruits. With the use of HPLC analysis we determined if,

and in what concentrations, α-solanine and α-chaconine occur in *S. mauritianum* and *S. anguivi* fruits, and compared their relative concentrations during four ripening stages in *S. anguivi*. Higher levels of toxicity in *S. anguivi* compared to *S. mauritianum* during the period of infestation by female fruit flies would support the prediction that stenophagous *Ceratitis* species, but not their polyphagous counterparts, dispose of a mechanism to cope with high levels of toxicity during larval development (Erbout et al., 2009). If so, this would provide additional evidence for the adaptive radiation hypothesis in this genus.

Fig. 1. Structures of α-solanine and α-chaconine



solanidine R=H

α – solanine R= glu, gal, rham

α – chaconine R= rham, glu, rham

Methods and Materials

Study Species and Reagents. Berries from both *Solanum* species were collected during May-June 2005 in Karura Forest, Nairobi, Kenya and identified by a researcher at the National Museums of Kenya (voucher specimens deposited). Reagents and alkaloids (α-chaconine and α-solanine) were provided by Kobian (Kenya) Limited (Nairobi, Kenya). All solvents used for chromatographic purpose were HPLC grade. Distilled water was prepared with a Millipore water purification system (Millipore, Bedford, MA, USA).

Alkaloid Extraction and Isolation. We extracted from freeze-dried *S. anguivi* berries at four consecutive ripening stages (stage 1: 36.8 g; stage 2: 34.2 g; stage 3: 70.05 g; stage 4: 40.63 g) and from *S. mauritianum* berries at ripening stage 1 (57.08 g). Freeze-drying, as compared to the use of fresh samples, reduces the risk of enzyme-catalyzed, wound-induced, and/or moisture-dependent compositional changes that may affect glycoalkaloid content (Stobiecki et al., 2003). Freeze-dried berries were crushed and extracted with MeOH at room temperature using protocols described in Trease & Evans (1978). Residues obtained after evaporation of MeOH *in vacuo* were partitioned between H_2O and C_6H_6-Et_2O (1:1). After addition of $KHCO_3$ to the aqueous layer, the latter was extracted with $CHCl_3$-EtOH (2:1). After evaporation of solvents *in vacuo*, alkaloids were chromatographed.

HPLC Analysis. MeCN stock solutions containing reference standards of α-chaconine and α-solanine were prepared for calibration. Samples were injected in triplicates, and each calibration curve was constructed by linear regression of analyte detector response (peak areas) to analyte concentration. UV spectra were recorded on a 168-diode array detector module attached to a Beckman System gold 126 HPLC system on a reverse phase C-8 silica (Ultrasphere C-8 column, 45 x 4.6 mm I.D., 5µm particle diameter, gradient elution with MeCN and H_2O mixture (23:77)). The flow rate was 1.0 ml/min and elution of the compounds was monitored at 232 nm. Each injection volume contained 10µL.

Results and Discussion

Results of the HPLC analyses are depicted in Fig. 1. Glycoalkaloid concentrations were higher in *S. anguivi* than in *S. mauritianum* berries during the first ripening stage, and substantially changed during ripening in the former. Although the used method allows separate isolation of α-chaconine and α-solanine, our results sometimes gave a mixture of both compounds. While concentrations were lowest during the second ripening stage and highest during the third one, they consistently decrease (to zero) during ripening (Table 1). In *S. anguivi*, both glycoalkaloids formed a variable but substantial fraction during each ripening stage, whereas in *S. mauritianum* they represented a small fraction of the total extraction only. The inverse relationship between glycoalkaloid concentration and fruit ripeness in *S. anguivi* supports the overall pattern observed in the majority of *Solanum* species (Carle, 1981), whereby a decrease in glycoalkaloid concentrations primarily results from a decline in the absolute amount of solasodine per fruit volume, probably due to enzymic degradation (Eltayeb & Roddick, 1985). Detailed enzymatic studies on *S. anguivi* (and most other *Solanum* species) are, however, required to confirm the true underlying mechanism(s) (Eltayeb et al, 1997).

Variation in glycoalkaloid composition between *S. anguivi* and *S. mauritianum*, as shown in this paper, may explain why polyphagous *Ceratitis* species can successfully reproduce in the latter host, but not in the former (De Meyer et al., 2002; Erbout et al., in press). The fact that stenophagous congeners such as *C. marriotti* and *C. venusta* successfully reproduce in *S. anguivi* (De Meyer et al., 2002) further suggests that these species, unlike polyphagous ones, can accommodate high levels of toxicity during larval development through (yet unknown) physiological mechanisms. Such conclusion, however, assumes that plant toxicity is a function of glycoalkaloid concentration. Whether, and to what extent, the relative mixture of glycoalkaloid may additionally determine host toxicity, remains to be examined.

Figure 2. HPLC chromatograms of total glycoalkaloid mixtures

*: α-chaconine and α-solanine; A: *Solanum anguivi 1* ; B : *Solanum anguivi 3* ; C : *Solanum mauritianum 1*

Peak areas can be used for quantitative assessment, which are obtained using a standard chosen so that it is eluted discretely and well separated from other components of the mixture.

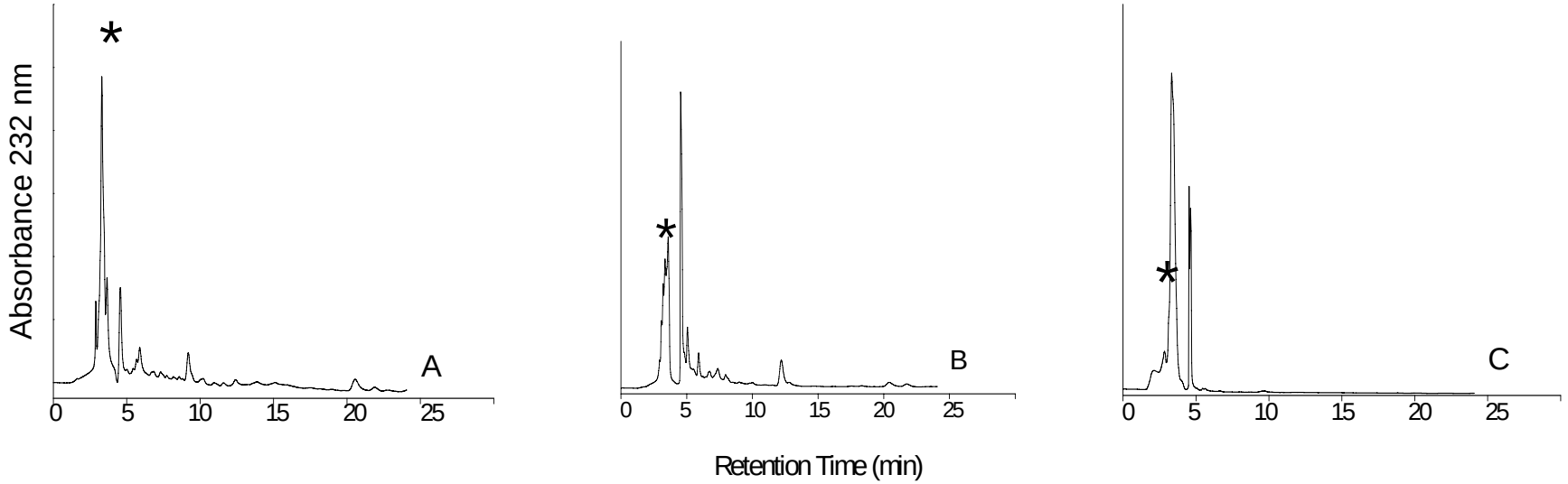


Table 1. Glycoalkaloid levels and relative peak area in *S. mauritianum* and in different ripening stages of *S. anguivi*

	µg(xy)/g fruit	***Peak area (xy)***	***Total peak area***	***% xy / TGA***
S. mauritianum	0.3	2.45	970.38	0.25
S. anguivi 1	11.9	46.59	394.77	11.8
S. anguivi 2	90.0	232.81	314.32	74.1
S. anguivi 3	49.0	248.79	1087.96	22.9
S. anguivi 4	18.4	69.37	765.55	9.06

xy: α-chaconine and α-solanine; TGA: total glycoalkaloid levels

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Chapter 3.3

Host plant toxicity affects developmental rates in a polyphagous fruit fly - experimental evidence

Abstract

Various, non-exclusive mechanisms have been invoked to explain the observed association between host plant use and speciation in insect species. In the Afrotropical genus *Ceratitis* (Diptera: Tephritidae), morphological, molecular, and biochemical data suggest that evolutionary radiation of stenophagous clades originates from their ability to exploit toxic hosts. To test if, and to what extent, development and fitness of *Ceratitis fasciventris*, a polyphagous congener known to infest over 43 host species, is adversely affected by host plant toxicity, we compared rates of development, survival and reproduction of captive bred individuals in four media that differ in alkaloid concentration. Despite reduced pupal and adult sizes, *C. fasciventris* larvae developing under low alkaloid concentrations successfully developed to the adult stage, probably as a result of accelerated pupation and ensuing restricted exposure to the toxic environment. High alkaloid concentrations, however, impaired their developmental process and prevented subsequent reproduction. The adverse effects of host plant toxicity on larval development in polyphagous fruit flies presented here, indicate that high alkaloid concentrations pose a significant constraint on host use by polyphagous *Ceratitis* species.

Introduction

Various, non-exclusive mechanisms have been invoked to explain the association between patterns of speciation and host plant use in phytophagous insects, such as shifts in - or expansion of - host plant use (Weingartner, Wahlberg & Nylin, 2006), hybridization (Schwarz *et al.*, 2007), adaptive radiation (Braby & Trueman, 2006) specialization and reproductive isolation (Schluter, 1998). A well-documented example of sympatric speciation associated with host plant use is provided by the monophagous apple maggot fly *Rhagoletis pomonella* (Walsh, 1867) (Diptera: Tephritidae) (Bush 1969, Berlocher 1998). The sibling taxa comprising the *R. pomonella* group are believed to originate from sympatric host shifts (Bush 1966), following the introduction of new host plants (e.g. *Malus pumila* (Miller)) and genetic changes in key loci responsible for host plant selection and (or) plasticity in diapause length (Feder *et al.*, 2003). Within the same taxonomic entity, the '*Lonicera* fly' probably evolved by hybridization between two native *Rhagoletis* species (*Rhagoletis mendax* (Curran, 1932) and *Rhagoletis zephyria* (Snow, 1894)) following a host shift to an introduced exotic weed (*Lonicera* spp) which catalyzed the local breakdown of reproductive isolation between both parental species (Schwarz *et al.,* 2007). Similar cases of speciation may have also been fascilitated by host use in polyphagous taxa such as butterflies (Murphy & Feeny, 2006; Mercader & Scriber, 2007), Hemiptera (Percy, Page & Cronk, 2004) and leaf-mining moths (Ohshima & Yoshizawa, 2005).

Expansion of host plant ranges with subsequent specialization on novel species, or re-specialization on ancestral ones, may be driven by behavioral, anatomical or physiological adaptations (Bush & Smith, 1998; Wheat *et al.*, 2007) that facilitate exploitation of resources that were previously present but unavailable within their natural ranges. In phytophagous insects, such adaptations are often associated with chemical defense mechanisms in plants (Ehrlich & Raven, 1964; Mitter, Farrell & Wiegmann, 1988; Bernays & Chapman, 2000). As an example, Wheat *et al.* (2007) showed that in Pieridae butterflies the detoxifying enzyme nitril-specifier protein (NSP) has a single evolutionary origin, appeared shortly after the origin of the

Brassicales hosts and was followed by a period of intense species radiation. Likewise, patterns of stenophagy in the Afrotropical genus *Ceratitis* (Diptera: Tephritidae), comprising over 90 fruit fly species that infest a wide range of unrelated host species, are associated with the toxicity of their host plants (Coates Palgrave 1983, Mabberly 1997). Morphological (De Meyer 2000; De Meyer *et al.*, 2005) as well as molecular (Barr & McPheron 2006) data confirmed that stenophagous species that infest the same host genus cluster into distinct monophyletic clades, while biochemical data (Coates Palgrave, 1983; Mabberly, 1997) confirmed that all host plant species within these clades contain high concentrations of toxic metabolites. Based on this congruence, the evolutionary radiation of stenophagous clades within the genus *Ceratitis* is believed to originate from the ability to exploit toxic hosts (De Meyer 2000, 2001).

To test if, and to what extent, development and fitness of polyphagous *Ceratitis* species are adversely affected by host plant toxicity, we compared infestation success and subsequent development and reproduction of captive bred *Ceratitis fasciventris* (Bezzi, 1920), a polyphagous species known to infest a wide range of host species, on three plant species (*Solanum anguivi* (Lamarck, 1794), *Solanum mauritianum* (Scopoli, 1788), *Mangifera indica* (Linnaeus, 1753)) that differ in level of toxicity, and one artificial substrate. Species of the plant genus *Solanum* (Solanaceae) typically differ in alkaloids concentrations: *S. anguivi* contains high concentrations of solasodine, alfa-chaconine and anguivioside (Honbu *et al.*, 2002). *S. mauritianum* contains similar glycol-alkaloids, however, in much lower concentrations, especially in ripe fruits (Erbout *et al.*, unpublished data). The unrelated species *Mangifera indica*, in contrast, contains carotene, thiamine, riboflavin, niacin, ascorbic acid, tryptophan, lysine and minerals (Goldsmith, 1976), but no alkaloids. While under natural conditions, *S. anguivi* is solely infested by stenophagous *Ceratitis* species of the *aliena* clade (De Meyer, 2000), *S. mauritianum* and *M. indica* are both natural hosts of *C. fasciventris* (De Meyer *et al.*, 2002). Based on presumed relationships between the presence of toxic metabolites and developmental pathways in holometabolous insects (Beebee & Bond, 1973; Thummel & Chory, 2002), we predicted a reduction in

developmental rate, developmental precision and individual size of F1 offspring, and in subsequent production of F2 offspring during the first five weeks of adulthood, with increasing alkaloid concentrations of their host plants.

Material and Methods

Study species and infestation

Ceratitis fasciventris is confined to Western and Central Africa where it is known to infest 43 different host species of 26 families (De Meyer, 2001; De Meyer *et al.*, 2002; Copeland *et al.*, 2006). Similar to other members of the genus, the life cycle of *C. fasciventris* comprises six stages: egg, three larval instars, pupa and adult. After developing into the third instar stage, larvae leave the host fruits and jump to the soil where pupation and subsequent emergence take place (Fletcher, 1989). The total time to adult development is approximately 25 days, however, each developmental stage may vary in duration (Papachristos, Papadopoulos and Nanos, 2008). For this study, parental individuals originating from a continuous breeding line regularly supplemented with congeners from wild populations were provided by the International Centre for Insect Physiology and Ecology (Nairobi, Kenya), where all experiments were carried out.

During the breeding experiment (see below), F1 individuals of *C. fasciventris* were reared on a non-toxic artificial medium and three host plant species that differed in level of toxicity: (i) *Mangifera indica* (MI, Anacardiaceae), a large evergreen tree originating from Southeast Asia (Mukherjee, 1972) does not contain glycoalkaloids and is the preferred host for several fruit flies including *C. fasciventris* (De Meyer *et al.*, 2002, Copeland *et al.*, 2006); (ii) *Solanum mauritianum* (SM, Solanaceae), also a preferred host, is a widespread invasive weed in Africa. Fruits contain glycoalkaloids such as solasodine (up to 185 μg (g^{-1}) dry weight in green berries; Drewes & Vanstaden, 1995); (iii) *Solanum anguivi* (SA, Solanaceae) is also widespread in

Kenya, does not act as host of *C. fasciventris* or any other polyphagous *Ceratitis* species, but is infested by the stenophagous species *Ceratitis marriotti* (Munro, 1933) *and Ceratitis venusta* (Munro, 1956) (De Meyer *et al*., 2002). Similar to other *Solanum* species, it contains alkaloids in all parts of the plant. While the composition of alkaloids is similar to *S. mauritianum*, concentrations in berries are significantly higher (> 1000 µg (g^{-1}) dry weight; N. Erbout, unpublished data). After locating fruiting plants of the three host species (larger Nairobi area) that were known to be regularly infested by *Ceratitis* species (Copeland *et al.*, 2006), ovaries of all fruits were netted to prevent field infestation. Upon collection, all fruits were individually measured and weighed.

Experimental design

The breeding stock of *C. fasciventris* which supplied parental (F0) individuals was housed under controlled climatic conditions (mean temperature: 28 ± 1°C; mean relative humidity: 50 ± 8%; photoperiod: L12:D12) in 50 cm x 50 cm x 50 cm cages (clear Perspex) with ample mesh windows for ventilation. Each cage contained ca. 1000 flies that were regularly supplied with water-soaked cotton wool, yeast hydrolysate (Torula yeast: hydrolysate powder mixed with sugar (Ekesi & Billah, 2006)), and a standard artificial infestation medium (further referred to as AM) consisting of 8.1% hydrolyzed yeast, 16.2% granular sucrose, 0.2% Methyl p-hydroxybenzoate, 24.2% carrot powder, 0.6% ascorbic acid and 50.7% water (Ekesi & Billah, 2006). At the start of the breeding experiment, seven days old males (20 ind.) and females (20 ind.) were retrieved from the breeding stock. They were housed under standardized climate conditions (see higher) in single cages (11cm x 11cm x 15cm), and supplied with either 200 g of ripe fruits (one of the three host species) or 200 g of AM (identical to that of the breeding stock and concealed in a perforated cling film). Each cage was provided with 4 g of yeast hydrolysate (see higher), and water ad libitum. Each treatment (host medium) was replicated over 20 cages, while four control cages that contained fruits but no flies per treatment were monitored to validate that fruits had not been infested in the field. Each adult fruit fly was allowed to

copulate and infest a single medium during 72 hours, after which fruits were removed and transferred to rearing boxes (12 cm x 15 cm x 20 cm) consisting of an upper compartment with a perforated bottom and fine-meshed top gauze where the fruits were placed, and a lower compartment supplied with sterilized sand (details in Ekesi & Billah, 2006). Rearing boxes were placed under standardized climate conditions (see higher) and checked daily. After leaving its natal fruit, migrating to the lower compartment and pupating, each pupa was individually housed and marked, weighed and measured after four days. Seven days after emergence, each F1 adult was crossed with a virgin individual of the same age but opposite sex derived from the continuous breeding line. Each pair was placed in a separate cage and provided with ample food and moisture under standardized climate conditions. Every four days, we provided 50 g of AM for infestation and counted the number of F2 offspring produced during the second till fifth week of adulthood (rearing procedure as described above). Hereafter, all adults were killed and preserved in 98% ethanol. Right and left wings, mid leg pairs and hind leg pairs of F1 adults were dissected, briefly immersed in glycerol and subsequently mounted on microscope slides in glycerol gelatin (details in Erbout, De Meyer & Lens, 2008).

Measurements and counts

We calculated total development time (pre-pupation: number of days between infestation and pupation; post-pupation: number of days between pupation and emergence) and emergence rate (not available for treatment MI due to logistic constraints) of 350 individuals per treatment. Lengths and widths of individual pupae were measured with a calibrated Leica WILD M3Z microscope, and volumes were calculated as length x width². Weights (to the nearest 0,1 mg) were obtained using a Mettler AT 261 (DeltaRange®) digital balance. Images of mounted adult limbs and wings were captured by a Leica MZ12s stereomicroscope (Leica Microsystems ltd., CH-9435 Heerbrugg, Switzerland) equipped with a Toshiba 3CCD camera and *Auto-Montage* software (Syncroscopy version 5.01, Synoptics ltd., Beacon House, Cambridge, UK). Optical magnification amounted to 10 x 6.3 for all leg measurements

and counts, and to 10 x 5.0 for all wing measurements and counts (additional optical magnification 2x). Linear measurements were performed with *Image Pro Plus* Version 4.1 software (Media Cybernetics, Silver Spring, MD, USA) using the *Best fit line* commando based of the number of pixels per line (one pixel per unit).

Fluctuating asymmetry was measured on 334 F1 adults (AM: 57♂, 73♀; MI: 55♂, 68♀; SM: 48♂, 33♀; no adults emerged from SA). In each individual, seven bilateral traits were measured, four of which were metric traits and three were meristic (selection criteria in Erbout *et al*., 2008). Metric traits comprised femur length of the second (fem2) and third leg pairs (fem3) and two distance measures between wing veins (vl1, vl2). Meristic traits comprised the number of setae on the caudal tibia side of the second leg pair (tib2) and on the rostral tibia side of the third leg pair (tib3), and the number of setulae on the ventral side of the R1 wing vein (vlst) (details in Erbout *et al*., 2008). Measurements and counts of either side of bilateral traits were made repeatedly and independently (sequence left-right-left-right or right-left-right-left) (Lens *et al*., 2002). Adult size was approximated as the femur length of the second leg pair averaged across sides and all repeated measures.

Statistical analysis

We carried out mixed regression analysis with Restricted Maximum Likelihood (REML) parameter estimation to separate real fluctuating asymmetry (signed FA; i.e. left minus right trait value corrected for trait size) from measurement error (Van Dongen, Sprengers & Lofstedt, 1999). The significance of FA was obtained from likelihood ratio tests while directional asymmetry (DA) was tested by *F*-tests with adjusted denominator degrees of freedom (Satterthwaite's procedure; Verbeke & Molenberghs, 2003). Following D'Agostino (1986) we compared the kurtosis of the signed FA values in each subsample against a critical *k*-value (details in Erbout *et al*., 2008). Unbiased individual FA estimates were calculated as deviations of random slopes from the fixed effects slope in the mixed regression model (Van Dongen *et al*., 1999), and standardized to zero mean and unit variance. To examine whether signed

FA values of different traits were correlated at the individual level (i.e. indicative for correlated development; Van Dongen *et al*. 1999), we calculated Pearson's correlation coefficients.

Variation in development (normal errors), mortality and reproduction (binomial trials) between treatments was tested with generalized linear mixed models in SAS version 9.1 (Littel *et al*., 1996). When testing relationships with FA, bilateral traits were treated as repeated measures at the level of the individual (CSH variance-covariance structure). Fixed effects were tested by *F*-tests (Littel *et al*., 2006), adjusting the denominator degrees of freedom by Satterthwaite's formula (Satterthwaite 1946). Random effects ('individual' and 'cage' in analysis of development; 'cage' in analysis of mortality and reproduction) were tested using Likelihood Ratio tests (Verbeke & Molenberghs, 2003). We used Chi-square tests to compare probabilities of pupal emergence and adult reproduction in relation to pupal weight (six classes: [0.002;0.003[, [0.003;0.004[, [0.004;0.005[, [0.005;0.006[, [00.006;0.007[, [0.007; 0.008] or two classes based on median traits values) and adult size (two classes based on median trait values). Interactive effects with host species were tested with Zelen's exact tests of homogeneity of odd's ratio.

Results

Developmental rate and size

A total of 404 pupae developed in 2002 g of host SA (20 pupae/100 g), 462 pupae in 2297 g of host SM (20 pupae/100 g), 1525 pupae in 2705 g of host MI (56 pupae/100 g), and 1312 pupae in 2050 g of the artificial medium AM (64 pupae/100g). Developmental times differed between hosts ($F_{2;65.6}$=3.3 p=0.04; no adults in host SA), both prior to ($F_{3;115}$=99.69 p<0.0001) and after pupation ($F_{2;64.6}$=24.07 p<0.0001) (Table 1; Fig. 1). Larvae pupated earlier in hosts SA and SM compared to MI and AM ($F_{1;112}$=197.43 p<0.0001) while pupae emerged later in SM compared to MI and AM ($F_{1;66.5}$=35.53 p<0.0001; no pupae emerged in SA) (Table 1; Fig. 1). Pupa lengths ($F_{3;97.4}$=9.94 p<0.0001), widths ($F_{3;95.2}$=11.62 p<0.0001) and volumes ($F_{3;94}$=11.45 p<0.0001) differed among the three hosts and the artificial medium, with significantly lower values in both *Solanum* species (length: $F_{1;93}$=13.07 p=0.0005; width: $F_{1;90.8}$=16.84 p<0.0001; volume $F_{1;89.3}$=18.22 p<0.0001) (Table 1). When comparing the distribution of pupa weights across six classes between host species SM and MI (no pupae emerged in SA), there was a higher proportion of lighter pupae produced in SM than in MI, both for the total number of pupae (χ^2_5=154.3, p<0.0001) and for the subset that subsequently emerged (χ^2_5=32.2, p<0.0001) (Fig. 2). After Bonferroni correction for multiple testing, average adult femur length differed between hosts ($F_{2;39.8}$=3.55 p=0.04), with significantly smaller values in SM compared to AM (p=0.049). The association between adult size and pupa volume ($F_{2;\,297}$=3.07 p=0.047) and adult size and pupa weight ($F_{2;305}$ =7.10 p=0.001) differed between hosts, with the strongest, positive correlation in SM.

Developmental instability

Mean FA levels did not significantly differ from zero in any of the 21 host-by-trait combinations, confirming that traits measured in this study did not show directional asymmetry (Table 2). The platykurtic distribution of the signed FA values did not

support the presence of antisymmetric individuals in any of the experimental groups either (Table 2; all *values* > critical *k*). Based on the comparison of REML values of models with and without random side effect, FA estimates were highly significant for all host-by-trait combinations (Table 2; all P<0.05). As signed FA values were not significantly correlated among traits (Pearson's correlation: all P> 0.05 after Bonferroni correction), the latter represented developmentally independent units and were not morphologically integrated. There was low covariation in standardized FA among different traits (χ^2_1= 0.006, p=0.58), indicating that single-trait FA only poorly predicted organism-wide asymmetry. After size correction, mean standardized FA did not significantly differ among the three hosts ($F_{2;28.6}$=1.41 p=0.26) also not when analyzing per trait and correcting for multiple testing (results not shown). There was, however, an overall significant negative correlation between FA and pupa weight ($F_{1;326}$=5.73 p=0.02). Across hosts, FA was not correlated with the total development time ($F_{1;93.7}$=0.73 p=0.4), nor with the development time prior to ($F_{1;78.7}$=2.59 p=0.11) or after pupation ($F_{1;274}$=0.84 p=0.4).

Reproductive rate

Both the probability of reproduction ($F_{2;42.46}$=3.4,1 p=0.04) and the average number of offspring ($F_{1;42}$=34.02, p<0001) differed significantly between hosts (Table 1). A total of 255 AM individuals, 313 MI individuals and 168 SM individuals produced offspring (Table 1; Fig. 2). While lighter pupae (i.e. lower weight than the host-specific median value) had a lower probability of emergence than heavier ones in both hosts (MI χ^2_1=67.8, p< 0.0001; SM χ^2_1=31.1, p<0.0001), the relationship was significantly stronger in MI than in SM (Zelen's test: p=0.0037). Adults that emerged from lighter pupae did not have a lower probability of reproduction than those emerging from heavier ones in either host (MI χ^2_1=0.85, p<0.36; SM χ^2_1=1.92, p=0.17). However, smaller adults (i.e. smaller than the host-specific median value) had a lower chance of subsequent reproduction than larger ones in host MI (χ^2_1=4.53, p=0.033) but not in SM (χ^2_1=1.75, p=0.19). This difference between hosts was confirmed by the Zelen's test (p=0.033). When pooled across hosts SM and MI, levels

of FA in fem2 ($F_{1;22.2}$=6.41, p=0.049) and tib3 ($F_{1;22.2}$=6.41, p=0.02) were inversely correlated with the number of offspring. The strength of this relationship did not differ between MI and SM ($F_{1;23.6}$=0.17 p=0.69).

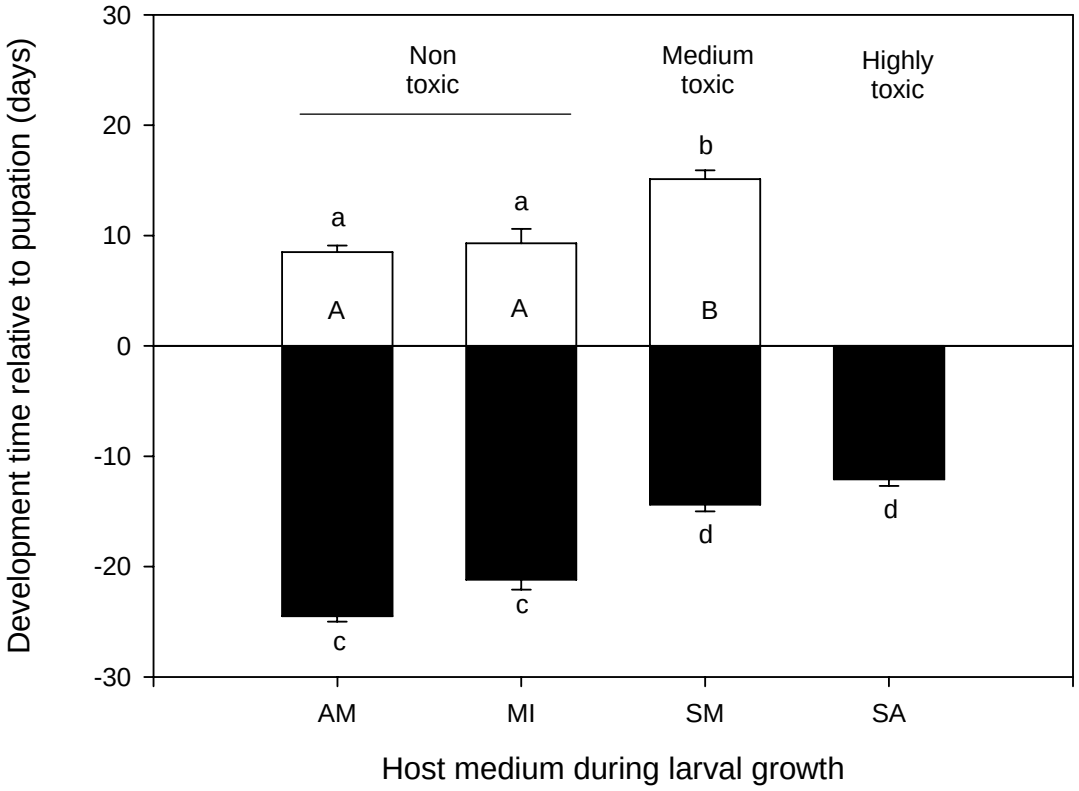

Figure 1. Development time in *C. fasciventris* in relation to host toxicity before (black bars) and after (white bars) pupation. Letters above bars indicate statistically significant differences in pre-pupal (a, b), post-pupal (c,d) and total (A, B) development time. AM= artificial medium; MI= *Mangifera indica*; SM= *Solanum mauritianum*; SA= *Solanum anguivi*.

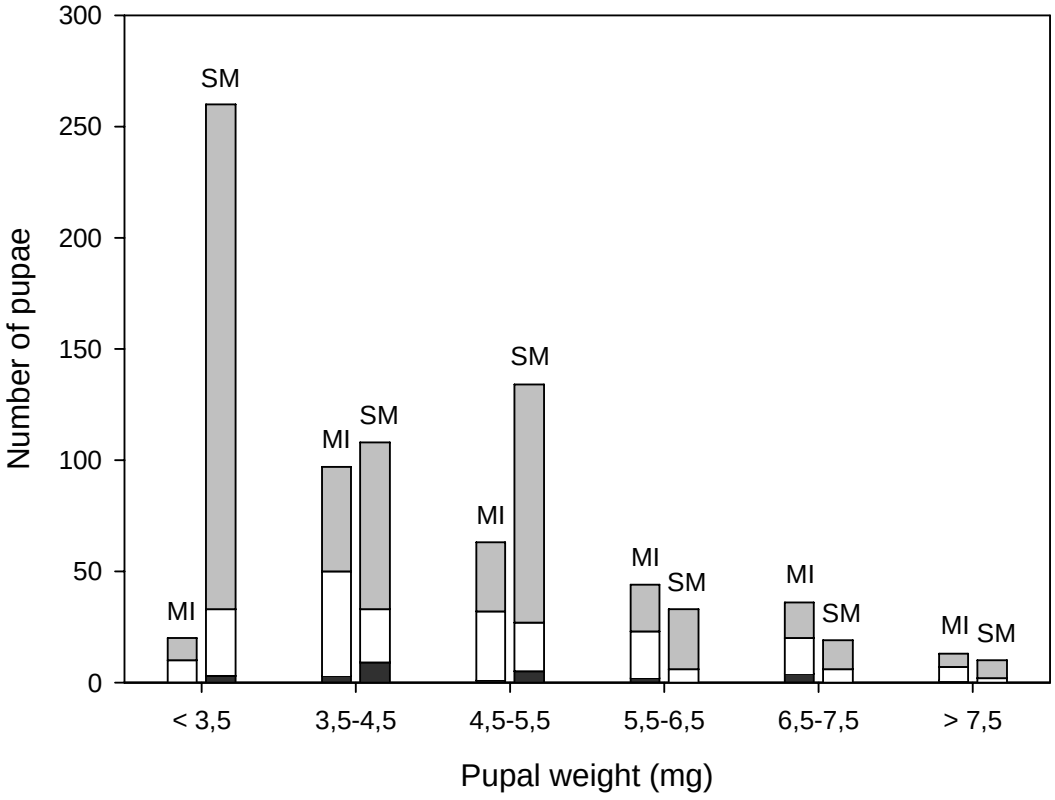

Figure 2. Emergence rate of *C. fasciventris* pupae in relation to pupal weight in a non-toxic (MI) and a medium toxic (SM) host . Light grey =non-emerged; white = emerged; black= emerged and producing offspring. MI= *Mangifera indica*; SM= *Solanum mauritianum.*

Table 1. Developmental and fitness traits (mean ± SE) of captive bred *Ceratitis fasciventris* in one artificial medium and three plant hosts.

	Developmental time			**Pupa traits**			**Adult traits**	
	pre-pupal (days)	**pupal (days)**	**total (days)**	**weight (mg)**	**volume (mm^3)**	**emergence (%)**	**probability of reproduction**	**number of F2 offspring/adult**
Artificial Medium	24.5 ±0.5	8.2 ±0.6	32.6 ±0.9	5.0 ±1.0	18.2 ±0.4	81.0	0.05 ±0.01	6.9 ±3.4
Mangifera indica	21.2 ±0.9	9.3 ±1.3	31.2 ±1.9	5.0 ±1.0	18.5 ±0.7	40.9	0.07 ±0.03	10.1 ±4.2
Solanum mauritianum	14.4 ±0.6	15.1 ±0.8	28.8 ±1.1	5.0 ±1.0	15.0 ±0.9	30.9	0.15 ±0.9	33.9 ±3.4
Solanum anguivi	12.1 ±0.6			8.0 ±1.0	17.5 ±0.4	0.0	0.0	0.0

Table 2. Variance components and distribution of signed fluctuating asymmetry of captive bred *Ceratitis fasciventris* in one artificial medium and three plant hosts.

Host	Trait[1]	N	F-test (num df, den df)	V_{FA}	V_{ME}	χ_1^2-test (d.f. = 1)	Kurtosis
Artificial medium	Fem2	121	0.49 (1,113)^{ns}	0.0005	0.0005	19.7**	26.14
	Fem3	115	3.19 (1,109)^{ns}	0.0009	0.0001	250.0**	12.88
	Tib2s	131	0.50 (1,124)^{ns}	1.1	0.2	132.0**	0.10
	Tib3s	131	0.67 (1,122)^{ns}	1.8	0.09	379.0**	0.87
	Vl1	123	0.05 (1,120)^{ns}	0.0002	0.00003	155.5**	15.74
	Vl2	122	0.09 (1,118)^{ns}	0.0009	0.00002	604.2**	6.90
	vlst	131	1.51 (1,127)^{ns}	1.9	0.04	589.2**	-0.24
Mangifera indica	Fem2	120	0.85 (1,114)^{ns}	0.0009	0.00003	463.8**	5.80
	Fem3	115	0.85 (1,104)^{ns}	0.0007	0.0004	43.4**	4.17
	Tib2s	121	1.43 (1,119)^{ns}	0.5	0.8	9.6**	0.21
	Tib3s	121	1.33(1,113)^{ns}	1.5	0.5	75.8**	0.44
	Vl1	119	2.28 (1,118)^{ns}	0.008	0.00002	1094.1**	53.47
	Vl2	118	0.63 (1,115)^{ns}	0.003	0.00001	422.8**	4.94
	vlst	121	0.62 (1,118)^{ns}	1.7	0.06	412.2**	0.14
Solanum mauritianum	Fem2	79	1.82 (1,75.1)^{ns}	0.0008	0.001	9.5**	3.78
	Fem3	78	0.1 (1,67.6)^{ns}	0.002	0.0004	64.1**	14.84
	Tib2s	82	0.11 (1,77.3)^{ns}	0.5	0.4	14.8**	0.17
	Tib3s	82	0.45 (1,74.1)^{ns}	1.8	0.4	76.9**	-0.57
	Vl1	81	2.47 (1,58.6)^{ns}	0.008	0.0001	802.3**	75.21
	Vl2	80	0.72 (1,76.2)^{ns}	0.0002	0.0005	4.3*	5.92
	vlst	82	0.37 (1,79.5)^{ns}	2.2	0.8	292.3**	1.46

[1]For detailed description see text; V_{FA}, variance in signed FA; V_{ME}, variance in measurement error; ns $p>0.05$; * $p< 0.05$; ** $p< 0.01$.

Discussion

C. fasciventris produced significantly less pupae per unit fruit under higher alkaloid concentrations. In the moderately toxic host, larvae pupated earlier than in the non toxic one, which reduced their exposure time to alkaloids. Pupae also emerged earlier, were on average smaller and contained a larger proportion of lighter individuals under moderate alkaloid concentrations. While lighter pupae had a lower probability of emergence in non-toxic and moderately toxic hosts, being smaller or lighter as pupae, or smaller as adult, had no adverse effect on reproduction in the latter. Despite evidence for environmental stress, average levels of fluctuating asymmetry in adults were not higher when raised in a toxic environment, contrary to our expectations. Under high alkaloid concentrations (as in *S. anguivi*), larvae pupated earliest, however, no pupae subsequently emerged.

Adaptations to cope with toxic plant metabolites were earlier demonstrated in *Pieris rapae* (Linnaeus, 1758), of which larvae detoxify the glucosinolate-myrosinase defense system of their host plants through a nitrile-specifier protein (Wittstock *et al.*, 2004; Wheat *et al*., 2007), and *Trichoplusia ni* (Hübner, 1803), of which larvae detoxify piperidine alkaloids through a combination of cytochrome P450-mediated metabolism and excretion of unmetabolized alkaloids (Castells & Berenbaum, 2008). Whereas similar detoxifying mechanisms have not (yet) been described within the genus *Ceratitis*, plasticity in the timing of pupation of *C. fasciventris* (this study) resulted in a reduced impact of toxic metabolites, while reproductive and developmental plasticity in the polyphagous congener *C. capitata* (Wiedemann) (Krainacker *et al.,* 1987), and in diapause length in monophagous or stenophagous members of the related genus *Rhagoletis* (Boller and Prokopy, 1976; Dambroski & Feder, 2007), also resulted in a better match with the physiology or phenology of their respective host plants.

In holometabolous insects with complex life cycles (CLC), such as fruit flies of the genus *Ceratitis*, development and growth are largely regulated by ecdysteroid-

Juvenile Hormone ratios (Žitňanová, Adams & Žitňan, 2001; Dubrovsky, 2005). Ingestion of steroid alkaloids, such as present in *Solanum* hosts, may interact with this balance and trigger early pupation (Thummel & Chory, 2002), as observed in *C. fasciventris* larvae developing in moderately toxic conditions. Under higher alkaloid concentrations, however, putative sustained levels of ecdysteroids may intervene with the Juvenile Hormone titer and thereby prevent pupal emergence (Dhadallia, Carlson & Le, 1998; Grebe, Rauch & Spidler-Barth, 2000), hence explaining why *C. fasciventris* larvae died in their exuvium after developing in *S. anguivi* fruits. Alternatively, reduced recruitment rates in *Solanum* host may result from nutrient deficiency due to restricted pre-pupal feeding or lower nutritional content, or from more intense competition due to larval crowding (Brévault, Duyck & Quilici, 2008). Whereas behavioral observations of adult females did not show differences in infestation rate between different host species (N. Erbout, unpubl. data), the total number of eggs laid per unit fruit could not be quantified. While proper data to test the nutritional or crowding hypotheses are hence lacking, pupae that developed as larva in moderately toxic conditions were, indeed, smaller and contained a larger proportion of lighter individuals than in non-toxic environments. To distinguish between putative mechanisms underlying these patterns, however, nutritional and physical properties of host fruits need to be decoupled from their alkaloid concentrations, e.g. by supplying fruits of different host species in a similar format (shape, texture, etc). As this inevitably implies manipulation and subsequent damage of fruits, changes in alkaloid concentrations due to oxidative and enzymatic reactions would hamper such comparisons (Finlay *et al.*, 1997).

C. fasciventris larvae raised in *S. mauritianum,* despite being smaller and lighter than pupae developing in non toxic environments, did not show reduced adult reproduction. While in various holometabolous insects nutrients used for reproduction are mainly obtained during adult feeding or stored at early larval stages, lipids are primarily accumulated during the final instar phase (Boggs, 1997; Shafiei, Moezek & Nijhout, 2001; Boggs & Freeman, 2005), and their content is believed to affect survival during the first four days after metamorphosis. In agreement with this, fecundity of *Speyeria*

mormonia (Boisduval, 1869) (Lepidoptera) remained unaffected by larval dietary restriction and decreased body mass (Boggs & Freeman 2005), while survival was reduced. Because in our experiment, individuals were provided with high quality food immediately after emergence, survival costs of developing in a toxic environment may have been (partly) masked. Alternatively, survival and fecundity may be highest at different protein-carbohydrate ratios, such as shown in *C. capitata* (Wiedemann, 1824) (Nestel & Nemny-Lavy, 2007; Lee *et al.* 2008), and if so, moderately toxic fruits may constitute a more favourable environment for reproduction by *C. fasciventris*. Irrespective of the true mechanism(s) underlying these patterns, smaller *C. fasciventris* pupae suffered less from reduced emergence and reproduction when developing under moderate alkaloid concentrations than in absence of the latter. Since larval growth in holometabolous insects is known to cease when the sequence of endocrine and physiological events initiated by the larval critical weight culminates in the secretion of ecdysteroids (Davidowitz, D'Amico & Nijhout, 2003), results from our study hint towards a reduction in larval critical weight level in moderately toxic environments.

While many studies showed adverse effects of environmental stress on individual and population levels of developmental stability (e.g. Parsons 1990, 1992; Polak and Trivers 1994; Møller & Swaddle 1997), *C. fasciventris* larvae developing in a moderately toxic host did not consistently show enhanced levels of fluctuating asymmetry as adults. Absence of significant relationships between pre-pupal stress and post-pupal indices of development may reflect adaptive decoupling (Moran, 1994), such as described in horn flies (*Haematobia irritans* (L.)) where exposure to sub-lethal concentrations of insecticide during the egg-phase was not reflected in increased developmental instability in adults (Da Silva, Mendes & Lomônaco 2004). In holometabolous species with a CLC, such as *Ceratitis* flies, adult traits are formed from cell lineages that remain undifferentiated until metamorphosis. Such compartmentalization may decouple the larval (instar) and adult stages and result in independent adaptation to temporally or spatially separated environments (Moran, 1994), e.g. by compensatory growth (Tomkins, 1999). Alternatively, absence of significant relationships between larval stress and adult FA may be due to differential

mortality, i.e. the fact that developmentally less stable (i.e. more asymmetric) individuals had a higher chance of dying before maturation (e.g. Floate & Fox, 2000). Strongly reduced pupation rates in moderately and highly toxic hosts, in combination with the lower emergence rate in the former, suggests that differential mortality prior to FA measurement may have masked relationships with FA. Despite this, individual levels of FA in one metric (femur length of the second leg pair) and one meristic trait (number of setae on the rostral side of the third leg pair) were inversely correlated with female fitness as measured by the number of offspring produced during the first five weeks of adulthood. Levels of FA in both traits were earlier shown to increase with genetic stress as a result of hybridisation (Erbout *et al*., 2008).

In conclusion, results from this study support the hypothesis that host plant toxicity affects developmental processes in a polyphaghous fruit fly. While *C. fasciventris* successfully reproduced in moderately toxic conditions, despite a reduction in size, higher levels of toxicity impaired its survival and reproduction. Further study is therefore required to understand how stenophagous congeners can successfully infest highly toxic host plants such as *Solanum anguivi.*

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Anubis baboon, Naivasha, Kenya
You were a worthy opponent in the battle for the berries

Section IV: Discussion

Collection site, on the way to Nanyuki, Kenya © Nathalie Erbout

Chapter 4.1

General Discussion

The evolutionary pathway leading to specialization in Tephritid flies is complex and factors shaping host plant use are highly diverse, with patterns spanning from extreme conservatism (Farrell et al., 1992) to extreme plasticity (Fitt, 1986a, 1986b). Hereto, a principal force guiding host range evolution is the phytochemical coevolution between the phytophagous insect and its host (Ehrlich and Raven, 1964), whereby host plant chemistry has been invoked in the explanation of both host plant use patterns of individual species and of patterns of insect species-radiation between different hosts. Ancestral tephritids most probably evolved from a saprophagous to a phytophagous lifestyle (Diaz-Fleischer et al., 2000). Herein, with respect to host range, four categories are acknowledged: monophagous, stenophagous, oligophagous and polyphagous, infesting on a host plant species, genus, family or multiple families respectively (Fletcher, 1989). As such, many fruit flies specialize in flowers and fruit structures that frequently lack the plant's characteristic secondary compounds (Mitter and Farrell, 1991), hence disputing the role of phytochemical coevolution in tephritids. Our results, however, have shown that *Ceratitis* species indeed use fruits containing plant characteristic secondary metabolites, and moreover, in case of stenophagous species, the host fruits prove to contain high concentrations of toxic metabolites (ms 2.2 and 3.2). Since our research has manly emphasized on the evolutionary patterns in the fruit flies, a possible role of chemical evolution in the host species remains unclear. Nevertheless, our results confirm a clear link between toxic host fruits and specialized *Ceratitis* fruit flies (ms 2.2 and 3.2). Moreover, our results suggest that the observed patterns in insect-host relationships should, from the host plant point of view, mainly be approached from an inter-species level, rather than from an intra-species level, of which the latter would be of count in phytochemical coevolution. After all, molecular phylogenetic patterns (ms 2.2) show that stenophagous flies group together according to toxic plant characteristic secondary metabolites shared by different plant species. In

this regard, molecular (ms 2.2) as well as morphological (De Meyer, 2005) phylogenetic data suggest that ecological similarities (hosts with common toxic secondary metabolites) and close relationship (common ancestry) reflect phenotypic or genetic similarities (in this case the ability to infest on toxic fruits), which is a characteristic for adaptive radiation (Givnish, 1997).

Evolutionary considerations of host use and specialization in *Ceratitis* flies

Host use in Ceratitis fruit fly species

Most species of tephritid flies, as is the case with most phytophagous insects, tend to have narrow host ranges (Jaenike, 1990). The genus *Ceratitis* however, includes 69 polyphagous species (out of 95) (ms 2.1). Some of them are extremely polyphagous infesting a wide variety of unrelated host plants. These are the main species considered as economic pests for the Afrotropical region (and outside the region for those established, such as *Ceratitis capitata* (Wiedemann, 1824). The 26 other species in the genus are specialized up to a certain level (plant species, genus, family). Biogeographic distribution patterns show (ms 2.1) that *Ceratitis* species are more abundant in moist areas, mostly forests. This latter reflects the host use pattern of *Ceratitis* flies, since they mostly infest fleshy fruits from trees, which are more profuse in these regions. Moreover, specialist *Ceratitis* species are more abundant in biogeographic regions around the equator, which are also the species richest (for both flies and host plants) and heterogenous regions. The wide variety of possible suitable host plants in these regions can thus provide many empty niches, a condition promoting radiation (Schluter, 2000). Furthermore, high fruit fly species richness and partial host range overlap (as is the case with many polyphagous fruit flies) can invoke inter-species competition, creating a need to seek and explore new open niches.

Host genera of the monophyletic clades of specialists described in ms 2.2 are rarely recorded as host of a polyphagous fruit fly, neither as host of other stenophagous species not included in the respective clades. This suggests a species specific adaptation to the main secondary metabolites of the respective host genera. Stenophagous species possibly evolved from a common ancestor that specialized on a host genus, by developing a mechanism that allowed them to cope with the genus specific secondary metabolites. Insects that are capable of detoxifying one class of plant compound usually cannot detoxify a very different class of secondary metabolites. From here, radiation occurred by specializing on different host species within the genus, with different species specific secondary metabolites. By following the strategy of adapting to new host plants within the genus, the fly could avoid competition.

Besides physiological adaptations when shifting to a new host, also behavioral adaptations are involved (Fitt, 1986a). Physiological adaptations or specializations, like coping with toxic metabolites, are mainly of relevance in the larval stage. On the other hand, host choice and host preference are behavioral aspects and in case of host shifts, this implicates an adaptation of the adult behavior. Females must first recognize and oviposit on a new host. Selection can than favor physiological adaptation in the larvae to toxic or nutritional factors. Our results (ms 3.3) have shown that the polyphagous fruit fly *C. fasciventris* under lab conditions displays infestation behavior when exposed to *Solanum anguivi*, a toxic species recorded as host of the stenophagous *C. marriotti* and *C. venusta*. However, no offspring, if any, successfully survived. Moreover, with few exceptions (http://projects.bebif.be/enbi/fruitfly), no records of polyphagous flies derived from stenophagous species hosts are known. It has to be pointed out though that the classification in polyphagous and stenophagous species is based on collection data whereby individuals were reared from their hosts and we can not make a distinction between the behavioral and the physiological capacity of the polyphagous species. Namely, we can not ascertain whether a polyphagous species has the physiological capacity, but lacks the behavior to successfully use toxic hosts.

Possible scenario's causing specialization in Ceratitis fruit flies

For the occurrence of the stenophagous lifestyle of those particular *Ceratitis* species and clades, several scenarios can be proposed. First, the host range of stenophagous species is limited due to lack of availability of other (polyphagous) hosts (i.e. ecological opportunity; Mardulyn et al, 1997; Gómez-Zurita et al, 2006), hence lack of opportunity to infest other hosts. This situation however is very unlikely since many examples in the field can be found where specialist and generalist hosts appear together (e.g. *Solanum anguivi* and *Solanum mauritianum*, Karura Forest, Nairobi, Kenya, pers. data). Second, the specialist species do not recognize other plants out of their range as potential hosts. Kühnle & Müller (2009) showed that differences in qualitative and quantitative composition of related metabolites can lead to differential plant acceptance, proving the complexity of plant cues and of insect responses that determine host acceptance behavior. Host selection by an extreme specialist is expected to be determined primarily by compounds restricted to its host plant, as opposed to ubiquitus compounds (Bernays, 2001). Fitt (1986a) showed that specialized *Dacus* (Tephritidae) species are quite host specific during oviposition and ignore other fruits, even when the preferred host is absent. The host preference had a genetic basis, resulting from specialized receptors which respond only to cues from appropriate hosts. In case of stenophagous *Ceratits* flies, these cues might be the species specific secondary metabolites. This would explain why members of, for example, the solanine clade, are restricted to one host species (*C. marriotti* and *C. venusta* restricted to *S. anguivi* and *C. aliena* and *C. turneri* restricted to *S. nigrum*), even though both plants belong to the same genus. The more common the secondary compound which the fly recognizes and considers suitable, the broader the host range can be. This host range can include specific host species from different families (as in the caffeine group, ms 2.2), or more closely related host species from one genus. This latter is the case for the majority of the flies as a straight result from the fact that most plant species contain family or genus specific secondary compounds that are not common in other host families (ms 2.2). Third, the parental stenophagous flies could actually persevere in a broader host range than recorded, with ability of larval survival, but whereby inter-

specific adult competition prevents successful production of offspring. This however, is very unlikely, because one could assume that during the extensive recording of *Ceratitis* fruit fly host use (Copeland et al., 2006) at least few records of other hosts of stenophagous species would subsist. Fourth, the actual host range of the specialists is more extended than noted, the adult flies may infest on other hosts (no behavioral limitation), but the larvae don't survive. This could be either due to maladaptation, nutrient deficiency or other physiological restrictions. This assertion however, stays presently indistinct.

Stress and development in *Ceratitis* fruit flies

Host plant secondary metabolites as a stressor for development

Many plant species pursue defence strategies against phytophagous insects through the production of secondary compounds that act as a repellent or impair the attacker's development (Howe and Jander, 2008). Secondary compounds are not universally found in higher plants, but are restricted to certain plant taxa, or occur in some plant taxa in much higher concentrations than in others (Fraenkel, 1959). Within their host range, insects are exposed to this immense variety of secondary plant metabolites, which comprise highly toxic compounds such as alkaloids, glucosinolates and furanucoumarines, among others (Schoonhoven et al., 2005). Many insects have various mechanisms of avoiding or detoxifying these compounds (Howe and Jander, 2008). Some specialist insects using the same plant taxon have evolved different detoxification or excretion mechanisms to avoid the impact of the same secondary plant metabolite (Ratzka et al., 2002; Wittstock et al., 2004). Hereby the abundance of plant secondary compounds provided a significant barrier for generalists to overcome, which has most possibly selected for adaptations in the specialist (Steppuhn et al., 2004). For fruit fly larvae of non- adapted species, these host plant secondary metabolites create a stressful environment during development. Our results showed several phylogenetic clades of stenophagous species (ms 2.2), infesting different host

plants with species-specific toxic secondary metabolites (ms 2.2; table 2), none of which are infested by polyphagous species. In order to test the possible effect of those toxic compounds on the development of a polyphagous *Ceratitis* fruit fly, a reliable quantification of these metabolites was inevitable. To do so, we compared the toxic glycoalkaloid concentrations between a polyphagous and a stenophagous host from the *Solanum* plant genus. Our results showed a significant difference, with very high values in *Solanum anguivi*, the stenophagous host. Hence, we applied these results in our developmental experiment, by using *Solanum anguivi* as a toxic environment to examine the effect of these high concentrations of toxic compounds, hence stressfactor, on the development of a polyphagous fruit fly.

Fluctuating asymmetry as a measure for developmental stability in Ceratitis

There is a wide interest in evolving a means of assessing the impact of environmental stressors on natural populations. Stressors of interest include pollutants to which organisms have never before been exposed as well as changes in natural environmental features such as temperature, humidity and shifts in resource use induced by global warming or habitat destruction. The demand for a broadly applicable bioindicator of stress is univocal. Here, fluctuating asymmetry (FA) has been postulated as a sensitive biomonitor tool in the field of conservation biology to detect the subtle effects of stress within a population (Clarke, 1995). Numerous examples of increased FA in natural populations exposed to environmental stressors exist in the literature (see Parsons, 1990; Clarke, 1993a). On the other hand, there are reports in which the anticipated increase in asymmetry was not found (Clarke, 1993b). Still, FA may be a sensitive indicator of the stress experienced by organisms during their development. Its use in this manner is a frequently proposed and potentially powerful tool but remains controversial partially because its underlying premise rarely has been critically tested. Such tests should include direct comparisons among individuals for which levels of FA, stress and fitness have been unambiguously quantified. In order to use FA as a tool to measure host-induced stress in *Ceratits*, we tested these premises in manuscript

3.1. Although we could not indicate an unequivocal link between FA and fitness, our results did show that FA can be used as an individual-based marker for developmental stress in *Ceratitis* flies.

Effect of host plant toxicity on development and fitness in Ceratitis

Our results supported the hypothesis that developmental processes in a polyphagous fruit fly are affected by the level of toxicity of the host plant on which their larvae develop. While fruit flies in a mildly toxic environment succeeded in successful development and reproduction despite their smaller sizes, higher toxic levels impaired their metamorphosis onto the reproductive lifestage. However, we did not found a significant relationship between larval stress and adult FA. As suggested (ms 3.3), this might be due to differential mortality prior to FA measurement or it might reflect adaptive decoupling (Moran, 1994). Although FA has been proven as a useful tool to accurately detect developmental disturbances in *Ceratitis* fruit flies (ms 2.1), our results suggest that several developmental characteristics, such as pupa size and weight and developmental rate, need to be explored to draw comprehensive conclusions regarding the effect of environmental stress on development. Despite this, individual levels of FA in one metric and one meristic trait were inversely correlated with female fitness as measured by the number of offspring produced during the first five weeks of adulthood. Although more data is desired to broadly confirm this relationship, it does suggest a putative role for FA as a measure for fitness. On the other hand, our results also imply that a smaller individual size due to environmental stress does not implicitly cause a reduction in fitness (measured by the number of offspring), which is often assumed. However, to generalize this result, more research is desired, preferably recording the individual life-span number of offspring as a measure of fitness. Validation of these findings would also explain why the polyphagous fruit fly *Ceratitis fasciventris* is so successful on *Solanum mauritianum* in the field (Copeland et al., 2006), despite the effect on individual size.

Possible mechanisms attending development: physiological considerations

Our results (ms 3.3) confirm that *C. fasciventris* is able to successfully develop and reproduce in the mildly toxic *Solanum mauritianum*, but not in the highly toxic *S. anguivi*, apparently as a result of advanced pupation, and hence, limited exposure to the plant secondary metabolites. Adaptations to toxic plant metabolites have been shown in the phytophagous butterfly *Pieris rapae* of which larvae became biochemically adapted to the glucosinolate-myrosinase defense system of their host by using a newly evolved detoxification mechanism (nitrile-specifier protein: NSP) (Wittstock et al., 2004; Wheat et al., 2007). Akin to this, the phytophagous moth *Trichoplusia ni* developed physiological resistance to piperidine alkaloids present in the highly toxic *Conium maculatum* (Castells & Berenbaum, 2008) by a detoxifying cytochrome P450-mediated metabolism and an efficient excretion of unmetabolized alkaloids. Our results indicate that, though *C. fasciventris* probably does not ply such a detoxifying mechanism, it is successful on mildly toxic hosts due to indirect physiological cues and a certain extent of plasticity in its developmental traits. As such, early metamorphosis in *C. fasciventris* as an escape mechanism from mildly toxic environments may be hormonally regulated i.e. triggered through the ingestion of steroid alkoloids that interact with the titter of the Juvenile Hormone (JH). Coordination and alternation between titers of JH and ecdysteroid are responsible for developmental transitions and induction of pupation and emergence in insects with complex life cycles (Žitňanová, 2001, Dubrovsky et al., 2004). Ingestion of advanced titers of alkaloids, transformed to body's own substances by *C. fasciventris* developing on *Solanum mauritianum*, may have altered the titer of JH, whereby inducing early pupation. A comparable transformation mechanism, albeit not directly related to toxic stress, was shown in *Drosophila melanogaster* (Thummel, 2002). This species is dependent on plant steroids as a source of cholesterol and is known to convert steroidal glycoalkaloids into ecdysone through a series of enzymatic steps.

Failure of *C. fasciventris* pupae to transform into adults in the highly toxic *S. anguivi* suggests that larvae died in their exuvium, probably due to an overdose of ecdysteroids interfering with metamorphic processes. Hereby the high doses of ecdysteroid prevent the pupated insect to reach the necessary level of JH to trigger emergence. Likewise, when experimentally treating *C. pipiens* mosquitoes with the insect growth regulator halofenozide, an insecticide, ecdysis was inhibited by this non-steroidal ecdysone agonist which disrupted the process of molting, after which larvae died within their exuvium (Boujelida et al. 2005). Such mechanism is also applied in insect pest control programs, where high concentrations of ecdysteroid mimics interfere with various aspects of the insect endocrine system (for review see Grebe 2000). Alternatively, pre-adult mortality of *C. fasciventris* under high alkaloid concentrations may have resulted from nutrient deficiency due to restricted pre-pupa feeding time in, or low nutritional content of, toxic fruits. While nutritional data to test this hypothesis are currently lacking, pupae that developed as larva in medium toxic conditions were also smaller, and contained a larger proportion of lighter individuals, than flies raised in a non-toxic environment. Contrary to our expectation, however, smaller and lighter pupae raised in *S. mauritianum* did not suffer from reduced adult reproduction. Despite their shorter development times, it is possible that *C. fasciventris* larvae did not suffer strong nutrient deficiency for reproduction, as phytophagous larvae were earlier shown to acquire most of their nutrients required for adult development and reproduction during the first two instar stages (Shafiei et al., 2001). In contrast, lipids required to survive the first four days after metamorphosis are mainly accumulated during the final instar phase. In a controled experiment on the effects on female fitness of larval semi-starvation in *Speyeria mormonia* (Lepidoptera), Boggs & Freeman (2005) showed that realized fecundity, but not survival, remained unaffected by larval dietary restriction and decreased body mass. In our experiment, *C. fasciventris* flies were provided with high quality food immediately after emergence. As a consequence, individuals were not challenged to locate new food resources during their first days of adulthood, and adverse effect of toxicity on survival may therefore have been (partly) masked in our study. Alternatively, mildly toxic fruits may have a more favorable nutritional composition for reproduction by *C. fasciventris*. In the related species *C. capitata*,

lifespan and fecundity were shown to be maximized at different protein-carbohydrate ratios (Nestel et al 2007; Lee et al 2007). Independent of the true underlying mechanism, smaller *C. fasciventris* pupae suffered less from reduced emergence and reduced reproduction when developing under mild alkaloid concentrations than in absence of the latter. Since larval growth stops when the sequence of endocrine and physiological events initiated by the larval critical weight culminates in the secretion of ecdysteroids (Davidowitz et al, 2003), results from our study suggest a reduction in larval critical weight level in mildly toxic environments. Once the larva attains the critical weight, the glands that synthesize and secrete JH, turn off, resulting in the drop in JH titer (Davidowitz et al., 2003). The combining effect of ecdysteroid increase and JH decrease trigger, in the case of *Solanum mauritianum* early, metamorphosis.

Chapter 4.2

General conclusion

Representatives of the Afrotropical genus *Ceratitis* display both generalist and specialist strategies in association with their host plants. Although widely distributed across the continent, the highest proportion of stenophagous (specialist) species occurred in subbiomes located around the equator. These regions are also characterized by the highest level of species richness. Molecular phylogenetic analysis supported previously proposed monophyletic lineages, whereby reconstruction of the ancestral character states for host plant relationships suggested that stenophagy (i.e. specialization on one host plant genus) evolved repeatedly and independently within the genus *Ceratitis*. Six clades comprised more than one stenophagous species that share host genera and genus-specific secondary metabolites, while at least in five different clusters a common polyphagous ancestor evolved into lineages with more restricted feeding preferences. We showed that representatives of one of the clades comprising stenophagous species are specialized on *Solanum* species. We confirmed that the *Solanum* host of the stenophagous species comprises much higher concentration of toxic secondary metabolites than the *Solanum* host of the polyphagous counterpart, wherein the concentration of these compounds were fractional. Experiments with polyphagous *Ceratitis* fruit flies exposed to these secondary metabolites, revealed that high levels of these toxic compounds constrain (prevent) their development, while mildly concentrations only reduced their individual size, but not their fitness. This implies that the high levels of toxic compounds of the stenophagous host plant form a barrier which can not be crossed by the polyphagous fly, preventing it of successfully using this plant as a host. Consequently this provides the stenophagous species, which can successfully utilize this host plant, with a possible unexploited niche, which creates opportunity for stenophagous species radiation. We conclude that the observed phylogenetic patterns of stenophagous *Ceratitis* clusters are most probably the result of an evolutionary process of ecological specialization to toxic hosts.

Perspectives for further research

In this thesis, only some aspects of the observed evolutionary and ecological patterns in the genus *Ceratitis* were investigated, and many questions remain to be answered in the setting of host specialization. Although we confirmed the relationship between stenophagy, radiation and host plant toxicity, some underlying mechanisms causing this pattern remain to be explored.

Fitness consequences of host-use in Ceratitis species

Although our results showed a clear fitness cost (no survival) for polyphagous fruit flies infesting stenophagous toxic hosts, the effect, and possible fitness cost for stenophagous flies infesting non-toxic plants remains unexplored. Due to logistic constrains, an experimental set-up to test this case failed, as rearing of stenophagous flies in the laboratory, proved not to be practically feasible. A continuous breeding line (which is desirable when performing such experiments) requires a continuous supply of infestation medium (host plant). Due to the specialized host use of stenophagous species, it is preferred to use the natural host plant, in order to avoid cryptic stress factors which may appear when rearing (if even possible) on other infestation media. Since most of these stenophagous hosts do not bear fruits all year-round, a continuous supply of media can not be assured. In theory, it would be possible to provide a year-round supply, since host phenology differs between different geographic regions. However, this would require a substantial logistic organisation, which was not possible in the scope of this research.

Behavioural aspects in host-use by Ceratitis species

Host plant selection by ovipositing females is a key process critical for the survivorship, performance, and fitness of their offspring (Thompson and Pellmyr, 1991). The mechanism underlying host choice is expected to be highly related to the

plant chemical composition (Jaenike, 1990). Ideally, we should have continuous breeding lines of both polyphagous and stenophagous species at our disposal. This would enable us to investigate infestation behaviour on toxic and non-toxic hosts within both groups. By performing choice related experiments, whereby both groups are offered both a toxic and a non-toxic host, we could investigate their infestation strategies. This could provide us with information of how specific both groups are in choosing their host. Also by offering only a non-(toxic) host to a stenophagous species, or a toxic host to a polyphagous species (ms 3.3), we can investigate the flies infestation behaviour in absence of their natural hosts. All these experiments should be performed on a substantial time scale, to exclude possible effects of postponed infestation behaviour. Further we could also compare infestation behaviour between stenophagous species within a clade, in order to investigate if mutual differences herein (next to other possible factors such as biogeographical, dispersal or detoxification differences) might have enhanced their radiation. For example in the *Solanum* clade four stenophagous species are two by two specialized on a certain *Solanum* species (ms 2.2). By offering three *Solanum* plant species as infestation medium (a non-toxic, a toxic from another stenophagous species from the same clade, and the natural toxic host), we could investigate the preference and the precision of the infestation behaviour of the stenophagous flies.

FAR-complex

Although *Ceratitis fasciventris, Ceratitis rosa* and *Ceratitis anonae* share a close phenotypic, genotypic and host profile resemblance, apparently no hybrids are encountered in nature. It would be useful to know whether there is any sterility or lower productivity, or any sex ratio distortion when rearing FAR hybrids in the laboratory, and whether the heterogametic sex in the hybrid offspring is more affected (Haldane's rule).

Effect of alkaloid concentration on Ceratitis development

A possible approach in future research, to be able to sparate nutritional, crowding and physical and chemical aspects affecting host choice and oviposition behaviour from the effects of different alkaloid concentrations, is to present a uniform artificial diet, to which increasing and known levels of alkaloids have been added.

Physiological aspects in host-use by Ceratitis species

Evolution of host plant chemical defences through development of secondary metabolites is believed to be closely followed by biochemical adaptations in phytophagous insects, whereby newly evolved detoxification mechanisms result in adaptive radiation of phytophagous lineages (Ehrlich and Raven, 1964). Various adaptive mechanisms have been proposed (Lambdon, 2001). However, such efficient mechanisms are often toxin-specific. The high defensive value of secondary plant metabolites has been demonstrated convincingly (Louda and Rodman, 1996; Mauricio, 1998), but their influence on the insect's physiology is less well understood. The true underlying mechanism which allows the stenophagous fruit flies to cope with the toxic secondary plant metabolites stays unclear. A possible mechanism involves cytochrome P-450 monooxygenase (P-450s), which plays a critical role in the detoxification of natural and synthetic toxins in a wide range of organisms (Nebert and Gonzalez, 1987). It has been shown that new P-450s can arise as phytophagous insects colonize different host plants and that interactions between the insects and their toxin-producing host plants have contributed to the diversification of the P-450 superfamily (Cohen et al., 1992). However, whether P-450s has played a role in the ability of stenophagous *Ceratitis* flies to cope with their host-specific toxic secondary compounds remains to be investigated.

Another possible mechanism underlying the capacity for phytophagous specialist flies to tolerate toxic metabolites is a microbial mutualistic relationship. Despite the ubiquity of mutualisms and increasing evidence of their ecological and evolutionary

importance, the role of mutualistic interactions in shaping evolutionary radiations has been largely unexplored (Futuyma, 2003). Such mutualistic interactions may interfere with the phytophagous insect's metabolism, enabling it to cope with toxic secondary metabolites. In cooperation with Dr. Anne Estes (University of Arizona), we currently investigate the possible existence of such mutualistic relationship in both polyphagous and stenophagous *Ceratitis* representatives.

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Summary

Using a multidisciplinary approach, my doctoral study aimed to describe the biogeography and phylogeny of host plant specialisation in phytophagous fruit flies of the genus *Ceratitis* (Diptera, Tephritidae) and to unravel mechanisms that underlie the evolutionary radiation of stenophagous (specialist) clades within this genus. In ms1 we highlighted some different aspects of insect-host relationships in phytophagous insects. We discussed different processes that play a part in the evolution of insect-host interactions. We conferred the aspects that determine host plant choice and selection, discussed which processes are known to enhance specialization and considered the role of chemical mediation in insect-host relationships. In ms2 we examined the extent at which Tephritidae species richness, relative proportion of host specialists and generalists and level of endemism differ among 23 sub-biomes distributed across the African continent, Madagascar and offshore islands, and the Arabian Peninsula, and explored relationships between patterns of species richness and host plant specialization. We concluded that the highest level of species richness and the highest proportion of stenophagous species are found in sub-biomes around the equator. In ms3 we reconstructed the phylogeny of the genus *Ceratitis* (Diptera: Tephritidae) by using molecular data from three protein encoding genes and 49 species (98 specimens) and investigated the evolution of host-plant specialization along the different recognized clades. Reconstruction of ancestral character states for host plant relationships suggested that stenophagy (i.e., specialization on one plant genus) evolved repeatedly and independently within the genus *Ceratitis.* Six clades comprised more than one stenophagous species that share host genera and genus-specific main secondary metabolites, while at least in five different clusters (*Podocarpus*, *Solanum*, *Strychnos*, *Tabernaemontana* and *Vespris* feeders), a common polyphagous ancestor evolved into lineages with more restricted feeding preferences. We conclude that the observed phylogenetic patterns for stenophagous *Ceratitis* clusters are the result of an evolutionary process of ecological specialization to toxic hosts. In ms 4 we tested the use of fluctuating asymmetry (FA) as an individual-based marker of developmental stress by comparing asymmetry levels in two parental *Ceratitis* species, *Ceratitis*

fasciventris and *Ceratitis rosa*, and their hybrid offspring. Although we could not indicate an unequivocal link between FA and fitness, our results did show that FA can be used as an individual-based marker for developmental stress in *Ceratitis* flies. In ms 5 we used HPLC analysis to ascertain the difference in concentration of two toxic secondary metabolites, α-chaconine and α-solanine, between a host plant (*Solanum anguivi*) of a stenophagous and a host plant (*Solanum mauritianum*) of polyphagous fruit fly. In ms 6 we tested if, and to what extent, development and fitness of a polyphagous *Ceratitis* fruit fly, *Ceratitis fasciventris*, is adversely affected by host plant toxicity by comparing rates of development, survival and reproduction of captive bred individuals on four artificial media that differ in alkaloid concentration. Results from this study supported the hypothesis that host plant toxicity affects developmental processes in a polyphagous fruit fly. While *C. fasciventris* successfully reproduced in moderately toxic conditions, despite a reduction in size, higher levels of toxicity impaired its survival and reproduction. As a general conclusion of this thesis we state that the observed phylogenetic patterns of stenophagous *Ceratitis* clusters are most probably the result of an evolutionary process of ecological specialization to toxic hosts.

Samenvatting

Insecten zijn één van de grootste aandeelhouders in de huidige globale biodiversiteit. Biotische en abiotische interacties tussen deze omvangrijke dierengroep en zijn omgeving dragen bij tot het behoud van deze biodiversiteit. Zo hebben mutualistische interacties tussen fytofage insecten en hun gastplanten een impact op vrijwel alle terrestrische ecosystemen en worden ze vaak verantwoordelijk geacht voor de omvangrijke soortenrijkdom. Inzichten in de evolutionaire en ecologische processen die deze associaties kenmerken kunnen dan ook een aanzienlijke bijdrage leveren tot het begrijpen van deze biodiversiteit. In dit doctoraat tracht ik via een multidisciplinair benadering de onderliggende mechanismen te onthullen die verantwoordelijk zijn voor de evolutionaire radiatie van gastheerspecialisten binnen een genus van fytofage fruitvliegen (*Ceratitis,* Tephritidae). In hoofdstuk 1 beschrijven we de verschillende aspecten van insect-gastheer associaties in fytofage insecten. We bespreken verschillende mogelijke evolutionaire processen die leiden tot specialisatie, gastheerkeuze en selectie, en bespreken de rol van chemische gastheer- kenmerken in dit alles. In hoofdstuk 2 onderzoeken we de hoe de variatie in soortenrijkdom en specialisatie verschilt tussen verschillende ecologische regio's. We concluderen dat de grootste soortenrijkdom en soortenspecialisatie voorkomt in regio's rond de evenaar. In hoofdstuk 3 reconstrueren we de fylogenie van het genus *Ceratitis* aan de hand van drie moleculaire merkers en onderzoeken de evolutie van gastheer specialisatie binnen verschillende monofyletische clades. Reconstructie van de ancestrale kenmerken voor gastheer associaties suggereert dat binnen het genus *Ceratitis* specialisatie herhaaldelijk en onafhankelijk is ontstaan. We besluiten dat het beschreven fylogenetische patroon van clusters samengesteld uit gespecialiseerde soorten het resultaat is van een evolutionair proces van ecologische specialisatie via de chemische constitutie van de gastheer. In hoofdstuk 4 testten we de bruikbaarheid van fluctuerende asymmetrie als individuele merker van ontwikkelingsstress. We vergelijken de mate van asymmetrie tussen twee polyfage fruitvliegsoorten en hun hybride nakomelingen. Hoewel we geen duidelijke link konden aantonen tussen fluctuerende asymmetrie en fitnes, duiden onze resultaten wel op de bruikbaarheid van

fluctuerende asymmetrie als individuele merker van ontwikkelingsstress. In hoofdstuk 5 vergelijken we aan de hand van een HPLC analyse de concentratie en samenstelling van de toxisch alkaloiden in twee gastheerplanten. We tonen aan dat de concentratie in een gastplant van een specialist significant hoger is vergeleken met de concentratie in een gastplant van een niet gespecialiseerde fruitvlieg. In hoofdstuk 6 testen we in welke mate de ontwikkeling en de fitnes van een polyfage fruitvlieg wordt beïnvloed door de chemische constitutie van een gastplant. We besluiten dat een matige concentratie aan toxische alkaloiden geen invloed heeft op de ontwikkeling, noch op de fitness, maar wel op de individuele grootte, en dat een hoge concentratie de ontwikkeling, overleving en reproductie belemmert. Als algemene conclusie van dit doctoraat kunnen we stellen dat de fylogenetische patronen van clusters van gespecialiseerde *Ceratitis* fruitvliegen het resultaat zijn van een evolutionair proces van ecologische specialisatie gestuurd door de chemische constitutie van de gastheer.